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Plant Responses to Bacterial *N*-Acyl L-Homoserine Lactones are Dependent on Enzymatic Degradation to L-Homoserine

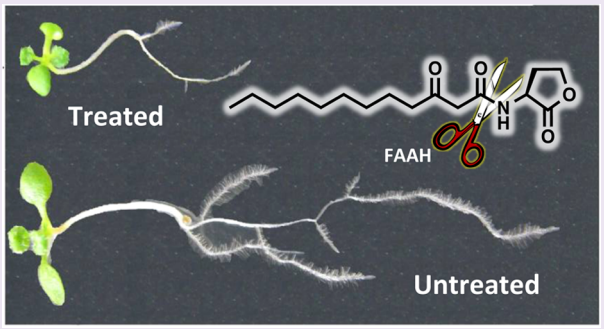
Andrew G. Palmer,[†,§,∥] Amanda C. Senechal,[†,§] Arijit Mukherjee,[‡,⊥] Jean-Michel Ané,[‡] and Helen E. Blackwell*[,†]

[†]Department of Chemistry, 1101 University Avenue, University of Wisconsin−Madison, Madison Wisconsin 53706, United States

[‡]Department of Agronomy, 1575 Linden Drive, University of Wisconsin−Madison, Madison Wisconsin 53706, United States

Supporting Information

ABSTRACT: Many bacteria use quorum sensing (QS) to regulate phenotypes that ultimately benefit the bacterial population at high cell densities. These QS-dependent phenotypes are diverse and can have significant impacts on the bacterial host, including virulence factor production, motility, biofilm formation, bioluminescence, and root nodulation. As bacteria and their eukaryotic hosts have coevolved over millions of years, it is not surprising that certain hosts appear to be able to sense QS signals, potentially allowing them to alter QS outcomes. Recent experiments have established that eukaryotes have marked responses to the *N*-acyl L-homoserine lactone (AHL) signals used by Gram-negative bacteria for QS, and the responses of plants to AHLs have received considerable scrutiny to date. However, the molecular mechanisms by which plants, and eukaryotes in general, sense bacterial AHLs remain unclear. Herein, we report a systematic analysis of the responses of the model plants *Arabidopsis thaliana* and *Medicago truncatula* to a series of native AHLs and byproducts thereof. Our results establish that AHLs can significantly alter seedling growth in an acyl-chain length dependent manner. Based upon *A. thaliana* knockout studies and *in vitro* biochemical assays, we conclude that the observed growth effects are dependent upon AHL amidolysis by a plant-derived fatty acid amide hydrolase (FAAH) to yield L-homoserine. The accumulation of L-homoserine appears to encourage plant growth at low concentrations by stimulating transpiration, while higher concentrations inhibit growth by stimulating ethylene production. These results offer new insights into the mechanisms by which plant hosts can respond to QS signals and the potential role of QS in interkingdom associations.



Quorum sensing (QS) is an intercellular signaling mechanism that allows bacteria to coordinate group behaviors in a cell-density dependent manner.[1,2] This signaling process is based on small molecule or peptidic signals called autoinducers. Bacteria constitutively produce autoinducers that diffuse or are transported out of the cell. As the bacterial population grows, the autoinducer concentration in the local environment likewise increases, and once a threshold concentration is achieved (and therefore population number), the autoinducers bind and activate their cognate receptor proteins.[3,4] This ligand/receptor complex then initiates the transcription of QS-regulated genes, allowing bacteria to display cell-density dependent phenotypes.

QS-regulated phenotypes vary broadly between bacterial species and their environments, and include bioluminescence, biofilm formation, motility, sporulation, root nodulation, and virulence factor production. Many of these bacterial group behaviors have profound effects on associated host eukaryotes. In the case of pathogenic bacteria, QS allows the bacteria to amass in sufficiently high number before initiating a coordinated attack on the host and overwhelming its defenses.[5] Indeed, QS mutants of the pathogen *Pseudomonas aeruginosa* are significantly less virulent in animal infection studies as compared to their wild-type counterparts.[6] Symbiotic bacteria, in contrast, use QS to instigate mutually beneficial relationships with their hosts at high cell densities.[7]

Gram-negative bacteria typically use *N*-acyl L-homoserine lactone (AHL) signals and their cognate LuxR-type receptors for QS (Figure 1A).[3] Our research laboratory and others have been actively involved in the development of non-native AHLs as tools to study QS in a range of Gram-negative bacteria.[8−13] One of our long-term research goals is to apply these agents to examine the roles of QS in prokaryotic−eukaryotic interactions.[9] As bacteria and their eukaryotic hosts have coevolved over millions of years, it is not surprising that certain hosts appear to be able to sense QS signals, potentially allowing them to alter QS outcomes.[14] Eukaryotes are known to display myriad responses to the AHL signals used by Gram-negative bacteria for QS. The native AHL signal of the pathogen *P. aeruginosa*, *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (OdDHL (**8**), Figure 1B) has seen some scrutiny in this regard, as it induces a wide variety of effects in mammalian cell lines. For example, transcriptome analysis shows that lung

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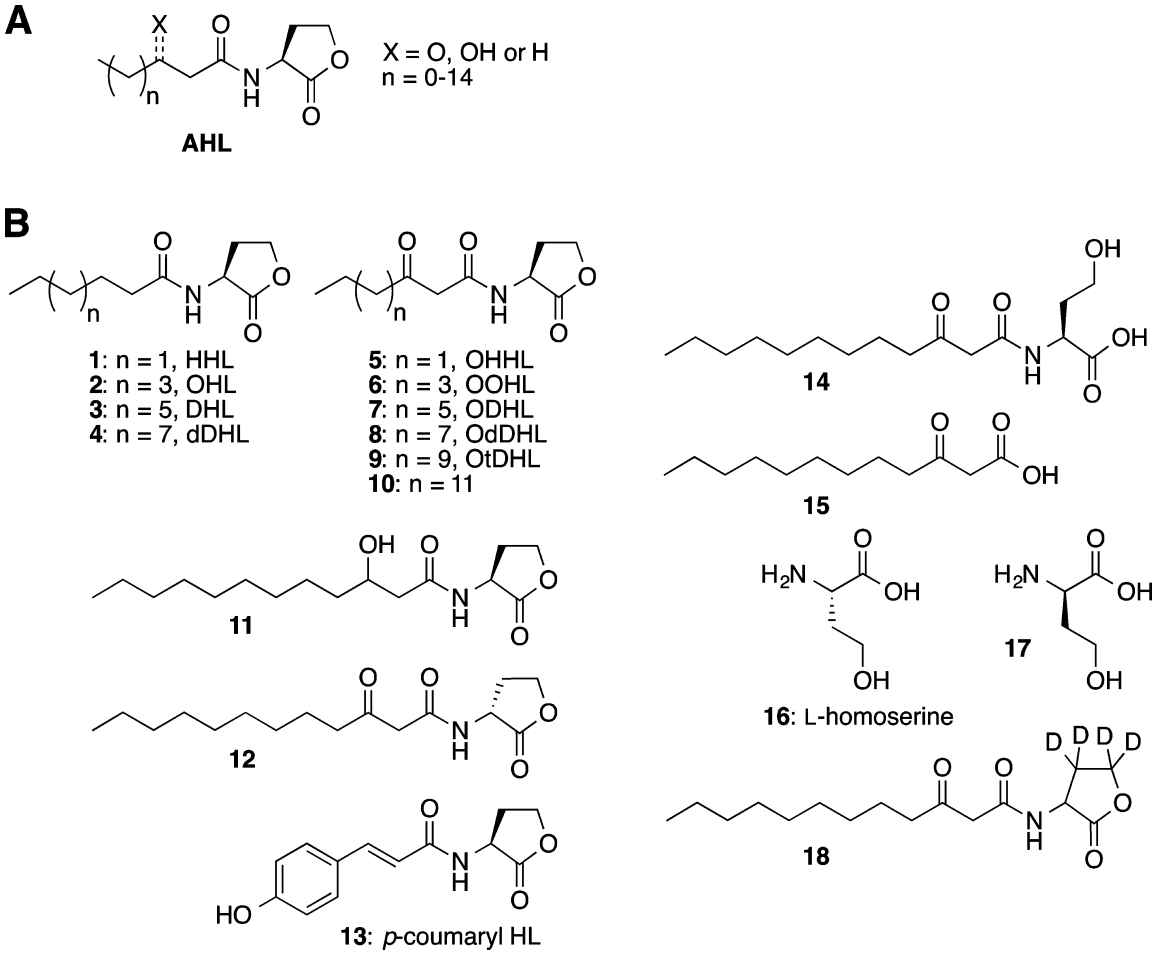

Figure 1. (A) Generic structure of acylated L-homoserine lactones (AHLs). (B) Structures of compounds evaluated in this study. Common abbreviated names for AHLs **1**–**9** listed for clarity.

epithelial cells display changes in the expression of 4347 genes in response to OdDHL.[15] This AHL is also known to elicit immunomodulatory responses in a broad array of mammalian cell lines, with reports of both immunostimulatory and immunosuppressant effects depending on concentration and cell type.[16−18]

To date, however, the responses of eukaryotes to AHLs have been more extensively characterized in plants as opposed to mammalian systems, perhaps due to ease of growth, genetic tractability, and the close relationships of plants with many Gram-negative bacteria (including pathogens, symbionts, and commensalists) in their native habitats.[19,20] Indeed, recent studies indicate that AHL-based QS is highly prevalent in the rhizosphere and endophytic communities of numerous plants.[21] Early documentation of plant responses to AHLs was reported in 2002, when Joint et al. showed that seaweed zoospores are able to detect AHL signals in seawater as a method of locating bacterial biofilms upon which to settle in marine environments.[22] Since then, evidence has emerged that AHL sensing by plants may be widespread.[14] For example, treatment of tomatoes with *N*-hexanoyl L-homoserine lactone (HHL, **1**; Figure 1B), an AHL produced by the tomato pathogen *Pseudomonas corrugata*,[23] causes increased resistance to leaf pathogens and induces production of defense-related and ethylene-inducible proteins.[24] Likewise, the model cress *Arabidopsis thaliana* displays changes in the expression of hundreds of genes in response to HHL, many of which are involved in responses to the phytohormone auxin.[25] HHL and other AHLs can also elicit profound changes to the root system architecture of *A. thaliana*.[25,26] Treatment of *Trifolium repens* (white clover) with OdDHL (**8**) results in increased transcription of elements associated with auxin-responsive and chalcone synthase promoters, which is consistent with a role for AHLs in elevating the production of auxin-associated and flavonoid synthesis proteins in plants.[27] Lastly, in the model legume *Medicago truncatula*, treatment with OdDHL or *N*-(3-oxo)-hexadec-11-*Z*-enoyl L-homoserine lactone (3-oxo-C16:1) induces changes in root protein production.[27] Adding further complexity to these observations, a subset of plant-associated Gram-negative bacteria have recently been shown to produce LuxR-type "orphan" or "solo" receptors, which lack a cognate AHL signal and instead appear sensitive to plant-derived signals (of unknown composition).[28] These findings suggest that there may be several interkingdom levels of regulation in plant−bacteria interactions.

Copious questions remain about the mechanisms and biological relevance of the measured responses of plants to AHLs to date. Analysis of past work is complex, as some of these studies report conflicting results, examined limited AHL concentration ranges (that may or may not be biologically relevant), and/or failed to characterize the actual chemical entities (i.e., AHLs or derivatives thereof) responsible for eliciting activity.[25,26] Notably, AHL degradation (via hydrolysis or enzymatic cleavage) is well-known to occur in the

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rhizosphere.[29] However, the role of these degradation products in plant responses to AHLs has not been examined. In addition, past studies have utilized racemic mixtures of AHLs,[25,26] despite the fact that bacteria do not produce D-AHLs. The specificity of certain plant response to AHLs has led some to hypothesize that an AHL receptor exists in plant systems; indeed, recent reports suggest that G-protein coupled receptors may be required for AHL response in *A. thaliana*.[30,31] Still, no definitive evidence of a specific AHL receptor in plants, or any other eukaryote, has emerged to date.

A more thorough understanding of how plants perceive and respond to AHLs is clearly needed. Herein, we report a systematic chemical biological and biochemical study of the responses of the model plants *A. thaliana* and *M. truncatula* to a variety of naturally occurring AHLs, non-native AHLs, and associated AHL degradation products. We evaluated compound responses in plants over a wide and biologically relevant concentration range, encompassing concentrations likely present in the bulk rhizosphere (1 nM to 10 μM) and higher concentrations that may be present in bacterial biofilms (>50 μM). Initial experiments revealed that AHLs exert concentration and acyl-chain length dependent effects on plant primary root length, with AHLs containing long aliphatic acyl chains (10−16 carbons) exerting the most prominent effects. Further growth studies revealed that the amide bond hydrolysis product, L-homoserine, mimicked the responses of plants to long-chain AHLs. We determined that fatty acid amide hydrolase (FAAH), an enzyme known for its ability to hydrolyze long-chain acyl amide substrates,[32] was a candidate enzyme for AHL hydrolysis. Subsequent experiments revealed that L-homoserine likely exerts its growth effects in plants through a combination of increasing transpiration and the production of the phytohormones ethylene and auxin. The work presented herein represents the most comprehensive analysis to date of the effects of AHLs in plants, and serves to illuminate, for the first time, a mechanism by which plants process biotic AHL signals. Further, as FAAH is common to diverse plant species,[33] our findings may represent a generalized mechanism by which plants perceive AHLs.

RESULTS AND DISCUSSION

Compounds Selected for Testing in Plants. We initiated our study by selecting a set of 17 AHLs and AHL degradation products for phenotypic screening in plants (**1**−**17**; shown in Figure 1B). AHLs **1**−**11** were chosen to examine the responses of plants to native AHLs with varying acyl chain lengths. Native AHLs share a common L-homoserine lactone "head" group, with LuxR-type receptor (and thereby often bacterial species) specificity conferred by the identity of the attached acyl "tail". These acyl tails are typically linear aliphatic chains of varying length (4−18 carbons) with differing oxidation states at the 3-positon (Figure 1A).[3] AHLs **1**−**11** represent the bulk of the known naturally occurring AHLs (Figure 1B), including those utilized by common plant and soil-associated bacteria such as *Burkholderia* spp. (**2**−**5**), *Pectobacterium carotovorum* (**5**), *Agrobacterium tumefaciens* (**6**), *Pseudomonas* spp. (**1**, **2**, **5**, and OdDHL (**8**)), and *Sinorhizobium meliloti* (**9**). While the saturated 16-carbon AHL (3-oxo-C16-HL, **10**) is not commonly produced by bacteria, the unsaturated 16-carbon AHL (3-oxo-C16:1) is utilized by species such as *S. meliloti* and *Agrobacterium vitus*.[34] 3-OH dDHL **11**, the primary AHL signal in *Acinetobacter baumannii*, was included as a representative native AHL with 3-OH functionality. We selected AHL **12**, the D-stereoisomer of OdDHL (**8**), to assess the signifiance of the lactone stereochemistry in eliciting responses in plants.

There can be exceptions to the canonical aliphatic acyl tail architecture in AHLs, as revealed by the recent discoveries of the native aryl HLs produced by *Rhodopseudomonas palustris* (*p*-coumaryl HL, **13**; Figure 1B) and *Bradyrhizobium* ORS278 (cinnamoyl HL).[35] Both of these bacteria commonly live in association with plants. We thus included the native *p*-coumaryl HL (**13**) in the current study; to our knowledge, the effects of this emerging class of native aryl HLs are yet to be tested in eukaryotes. The responses of plants to *p*-coumaryl HL (**13**) are of additional interest because we have found many structurally related, synthetic aryl HLs can strongly modulate LuxR-type receptors in a range of Gram-negative bacteria.[8−11]

We included the ring-opened AHL (**14**), 3-oxo-dodecanoic acid (**15**), and L-homoserine (**16**) in our experiments to investigate the effects of AHL degradation products on plants (Figure 1B). These three products are derived from the common AHL, OdDHL (**8**). Hydrolysis of the lactone (as in **14**) typically occurs relatively slowly under aqueous conditions (half-lives of ∼12−48 h at pH ≈ 7[36]), resulting in a QS-inactive compound. As typical plant assays usually extend over several days, it was prudent to include this hydrolysis product in our studies. Enzymes capable of hydrolyzing the AHL amide bond have been identified in both prokaryotes and eukaryotes, which provided an impetus for the inclusion of the amide hydrolysis products (**15** and **16**).[37,38] We thus also examined D-homoserine (**17**) in order to compare its activity to its potential hydrolysis precursor, D-OdDHL (**12**). Collectively, this set of compounds represents the largest collection of AHLs and related derivatives examined to date for effects on plants.

AHLs and Their Degradation Products Modulate Primary Root Length. A number of prior studies with model plant systems (e.g., *A. thaliana* and *Nicotiana tabacum*) have utilized primary root length as a phenotypic marker for the growth-modulatory effects of exogenously applied compounds, including AHLs.[26,39,40] We therefore utilized this straightforward assay to evalute the effects of compounds **1**−**17** on *A. thaliana* seedlings. The seedlings were examined following 12 days of exposure on media supplemented with a single, initial dose of compound at concentrations ranging from 10^{-12} to 10^{-4} M (see Methods). This wide range encompasses the AHL concentrations associated with the bulk rhizosphere (1 nM to 10 μM) as well as those purported to be present in bacterial biofilms (>50 μM), which can form on plant roots.[41]

We found that the AHLs elicited a biphasic growth response in *A. thaliana* seedlings, with increased primary root length observed at submicromolar concentrations of compound and a significant reduction in root length observed at concentrations ≥50 μM. A dose response analysis for OdDHL (**8**) is shown in Figure 2A, and assay data at low and high concentrations for all 17 compounds are shown in Figure 2B (for complete dose response analyses, see Supporting Information Figure 1). Representative images of an *A. thaliana* seedling exposed to 100 μM OdDHL (**8**) and an untreated seedling are shown in Figure 3. Normal growth and development were restored in seedlings treated with 100 μM **8** for 14 days by transferring them to AHL-free growth media; separate assays revealed that the stunted root growth was not simply a result of toxicity (see Methods). Notably, similar AHL-induced biphasic growth effects were observed in *M. truncatula* (Supporting Information Figure 3), demonstrating that these responses are not limited to *A. thaliana*.

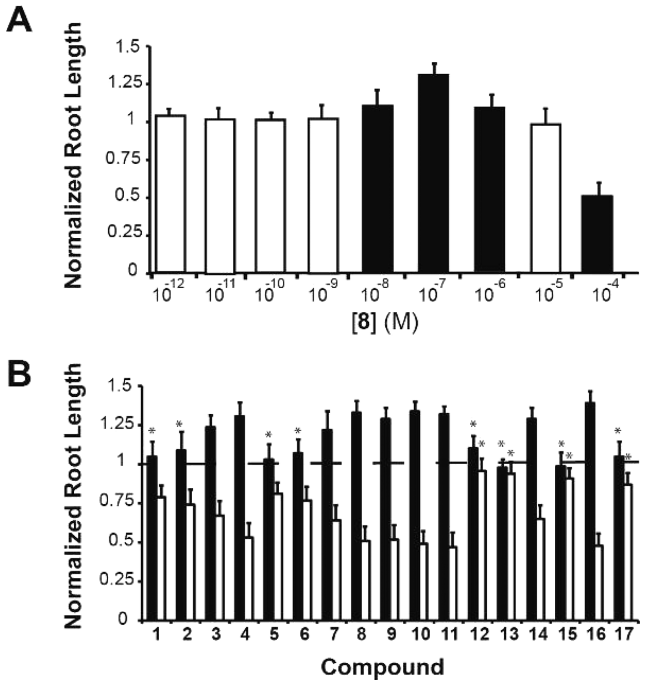

Figure 2. *A. thaliana* seedling growth assay data. Wild-type plants were exposed to the indicated compounds for 14 d and evaluated for variations in root length. The results were normalized to the root lengths of untreated samples (see Methods). Data shown are the average of 30 samples. (A) Plot of a dose−response evaluation of the effects of OdDHL (**8**) on primary root length. Entries with $p < 0.05$ shown with black bars. (B) Plot of effects of compounds on primary root length at 0.1 μM (black) or 100 μM (white). These two conditions were selected to juxtapose the effects of low concentration and high concentration treatments on seedlings. Unless noted by *, all entries have $p < 0.05$ relative to untreated samples.

Figure 3. Images of wild type, 12-day old *A. thaliana* seedlings grown on untreated solid MS media (left) or media containing 100 μM OdDHL (**8**) (right). OdDHL (**8**) results in a significant decrease (2−3 fold) in primary root length at this concentration.

The observed increased and decreased root elongation effects upon AHL exposure are generally consistent with prior studies by von Rad et al. and Ortíz-Castro et al. in *A. thaliana* and *M. truncatula*.[25,26] It is likely that the biphasic response to AHLs has not been previously noted because these prior studies focused on only one AHL concentration regime (submicromolar[25] or mid- to high micromolar[26]). However, we failed to observe the highly branched lateral root architecture reported by Ortíz-Castro et al. in either *A. thaliana* or *M. truncatula* upon treatment with decyl HLs (**3** or **7**) at high concentrations.[26] We found that *A. thaliana* sensitivity to aliphatic AHLs increased with acyl chain length, with the effects strongest for AHLs with tails of 12 carbons or longer (e.g., **8**−**11** Figure 2B). Sensitivity appeared to be primarily dependent upon acyl chain length, as dDHL (**4**), OdDHL (**8**), and 3-OH dDHL (**11**), which are identical except for the oxidation state of the 3-position carbon of the acyl group, were equally active in eliciting growth responses. We also note that lactone stereochemistry played an important role in the *A. thaliana* growth response, as AHL **12**, the D-enantiomer of OdDHL (**8**), failed to cause significant growth effects. Treatment with aryl HL **13** also caused no significant response in *A. thaliana* seedlings, in contrast to the growth effects observed for the aliphatic AHLs (Figure 2B and Supporting Information Figure 1).

Turning to the AHL degradation products, we found that OdDHL (**8**) and its hydrolyzed analogue **14** elicited similar growth modulatory effects in *A. thaliana* (Figure 2B), suggesting that AHL effects on plant growth do not require an intact lactone. Similar effects were also observed for OHHL (**5**) and its hydrolyzed analogue in *A. thaliana* (data not shown). This result contrasts with the known structural requirements for AHLs to bind to LuxR-type receptors in bacteria, where the lactone is essential. More interestingly, *A. thaliana* and *M. truncatula* seedlings exposed to increasing concentrations of the AHL amide bond hydrolysis product, L-homoserine (**16**), displayed a biphasic growth pattern that closely approximated that of dDHL (**4**), OdDHL (**8**), 3-OH dDHL (**11**), and the lactone hydrolysis product **14**. Consistent with the AHLs, the growth modulatory response of L-homoserine (**16**) was dependent on stereochemistry, as D-homoserine (**17**) elicited responses similar to the D-analogue of OdDHL (**12**). The growth effects observed for L-homoserine (**16**) were also comparable to those for the cyclized variant, L-homoserine lactone (data not shown). The other AHL amide bond hydrolysis product, 3-oxo-dodecanoic acid (**17**), had no significant effect on root length at the concentrations evaluated, suggesting that this component was not an active agent.

***At*FAAH Hydrolyzes AHLs in an Acyl Chain Length Dependent Manner.** Given that the acyl chain appeared to be the major structural feature of the AHLs that influenced plant growth and development, we were surprised at the comparable activity of L-homoserine (**16**) to **4**, **8**, **11**, and **14**. Background (uncatalyzed) amide hydrolysis was unlikely to be a concern.[42] We therefore considered the possibility that plants produce one or more enzymes capable of catalyzing the hydrolysis of the AHL amide bond—for example, an amino hydrolase—to give L-homoserine (**16**), which was then the active agent in plants.

In an earlier study, Ortíz-Castro et al. noted that AHLs with long aliphatic acyl chains are similar in structure to *N*-acyl ethanolamines (NAEs), the native substrates for Fatty Acid Amide Hydrolases (FAAHs).[26] Both AHLs and NAEs possess a hydrophobic acyl chain and a more polar headgroup separated

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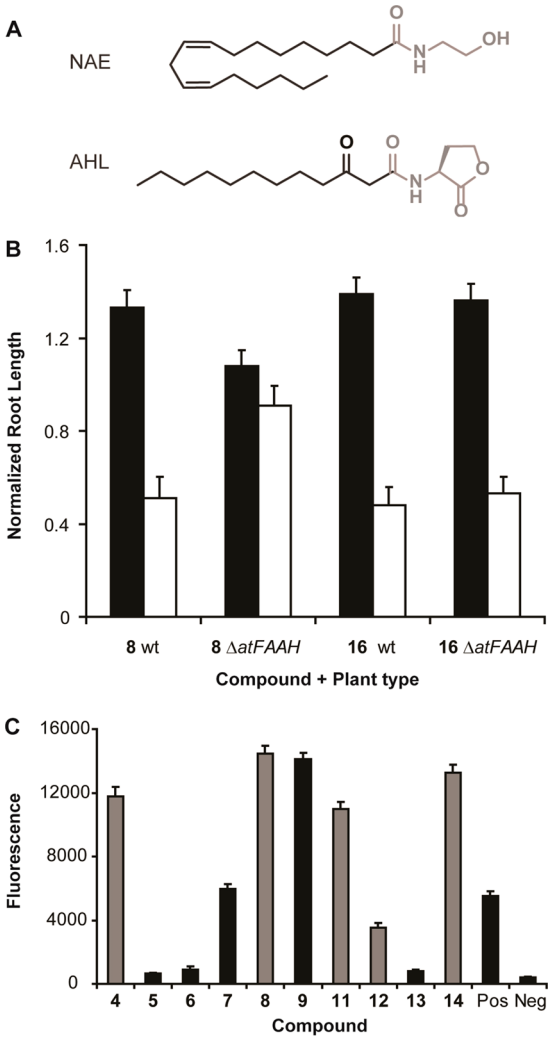

Figure 4. A) Structures of a representative NAE (linoleoyl ethanol amide) and AHL **8** (OdDHL). Similar structural features are shown in black (long aliphatic chains) and gray (polar amide head groups). (B) Root length growth effect data for *A. thaliana* (wt) or the *A. thaliana* (Δ*AtFAAH*) mutant treated with OdDHL (**8**) or L-homoserine (**16**) at 0.1 μM (black) and 100 μM (white). Results shown are the average of 30 samples. (C) AHL hydrolysis data for the indicated compound (at 100 μM) by purified *At*FAAH as monitored by the reaction of L-homoserine (**16**) with fluorescasmine. Compounds **4**, **8**, **11**, **12**, and **14** all have a 12-carbon acyl tail and are indicated in gray for comparison (note, **12** is the D-enantiomer of **8** (OdDHL)). Pos (Positive control) = linoleoyl ethanolamide (100 μM); Neg (Negative control) = DMSO.

by an amide bond (Figure 4A). Like AHLs, NAEs can alter root system architecture, with the exogenous addition of NAEs (>10 μM) significantly reducing root elongation.[43] Members of the FAAH family, known to be common in animals, are responsible for cleaving the amide bond in NAEs and rendering them inactive as endogenous signaling molecules. FAAH homologues have also been identified in diverse plant species, including *A. thaliana* and *M. truncatula*, and are believed to act upon endogenous plant NAEs in a manner analogous to their mammalian counterparts.[43,44] Studies of FAAH in *Arabidopsis thaliana* (*At*FAAH) indicate that these proteins are produced in the roots throughout the early stages of plant growth (≤14 days),[43] consistent with the time period of the assays in the current study. Also, FAAH is known to be membrane-associated and acts upon ligands present in the cellular environment.[32] As plant cells are permeable to AHLs,[45] we reasoned that FAAH-catalyzed hydrolysis of the AHL amide bond could occur in plants, to yield L-homoserine (**16**). In 2008, Ortíz-Castro et al. tested the general hypothesis that plant FAAHs can process AHLs;[26] the authors reported that *A. thaliana* FAAH deficient seedlings (Δ*AtFAAH*) were hypersensitive to decyl HL (**3**; Figure 1B) in primary root growth assays (at midmicromolar concentrations), while seedlings overexpressing *AtFAAH* were more tolerant. However, these past results directly contradict our hypothesis, as we predicted that the *loss* of *AtFAAH* would correlate with *reduced* sensitivity to AHL (and thereby L-homoserine (**16**)).

To reconcile these prior experimental results and further explore our hypothesis that L-homoserine (**16**) was an agent responsible for AHL-induced growth effects in plants, we investigated the activities of OdDHL (**8**) and L-homoserine (**16**) in *A. thaliana* FAAH deficient seedlings (Δ*AtFAAH*) using the root growth assay described above. As shown in Figure 4B, root elongation in the Δ*AtFAAH* mutant was insensitive to both 0.1 and 100 μM OdDHL (**8**) exposures, exhibiting no significant increase or decrease in total length at these concentrations, respectively. However, in response to L-homoserine (**16**), these mutants displayed a comparable biphasic response as wild-type *A. thaliana*, with both significantly increased (0.1 μM) and decreased (100 μM) root elongation clearly observable. These results in the Δ*AtFAAH* mutant are consistent with our hypothesis that AHL cleavage by FAAH, yielding the active L-homoserine (**16**), is necessary for AHL-induced growth responses in *A. thaliana*.

While these results support AHL degradation as being crucial in plant responses to these signals, it remained to be shown whether AHLs could actually serve as substrates for *At*FAAH. We therefore evaluated AHL amide hydrolysis by *At*FAAH *in vitro* using the purified enzyme. As this hydrolysis reaction would yield a primary amine (i.e., L-homoserine (**16**)), we utilized fluorescamine, which only fluoresces after reaction with an amine,[46] to assess the extent of AHL cleavage by FAAH (see Methods). Fluorescamine has previously been established to react with a variety of amino acid monomers, and preliminary tests confirmed an efficient reaction of fluorescamine with L-homoserine (**16**). As shown in Figure 4C, the NAE linoleoyl ethanolamide, a known substrate for *At*FAAH,[43] caused a significant increase in fluorescence after incubation with purified enzyme for 30 min and addition of fluorescamine, confirming the viability of using this assay to measure *At*FAAH activity. We screened AHLs **4**–**9** and **11**–**14** (encompassing acyl chain lengths from 6–14 carbons) in this assay (at 100 μM), and the results demonstrated that the level of AHL amide hydrolysis by *At*FAAH (as determined by increased fluorescence) was positively correlated with acyl chain length (Figure 4C). Hydrolysis of the AHL amide appeared to be only mildly affected by the functionality at the 3-position, as AHLs **4** and **11** were 70–80% as active as OdDHL (**8**) in this *At*FAAH cleavage assay. We initially hypothesized that the lactone headgroup of the AHLs might interfere with FAAH activity; however, **14**, the lactone-hydrolyzed analogue of OdDHL (**8**), displayed approximately 90% the activity of **8**, suggesting that this effect was minimal in our assay. The D-enantiomer of OdDHL (**12**) was 30% as active as its L-enantiomer (**8**), while AtFAAH did not cleave the aryl HL **13** to any appreciable extent. These *in vitro* assay results with purified *At*FAAH and

AHLs **4**–**9** and **11**–**14** largely corroborate the data trends observed in the primary root elongation assays above.

AHLs Stimulate Transpiration. We next turned our attention to the mechanisms by which accumulating L-homoserine (**16**) might alter plant growth and development, in order to develop a more thorough understanding of the observed growth effects. Prior studies have established that the exogenous addition of L-homoserine (**16**) at submicromolar concentrations stimulates opening of leaf stomata in *Phaseolus vulgaris* (common bean), which increases the rate of transpiration (water loss).[47] Increased transpiration encourages water and nutrient uptake and can improve photosynthetic efficiency, thereby stimulating plant growth.[48] We therefore reasoned that AHLs could stimulate plant growth after their cleavage via *At*FAAH (to yield L-homoserine (**16**)) by increasing transpiration. To test this hypothesis, we evaluated the effects of OdDHL (**8**) and L-homoserine (**16**) exposures (at 0.1 and 100 μM) on *A. thaliana* transpiration by monitoring the loss in leaf weight (due to water loss) as a function of time.[49] As shown in in Figure 5, treatment of wild-type *A. thaliana* with **8**

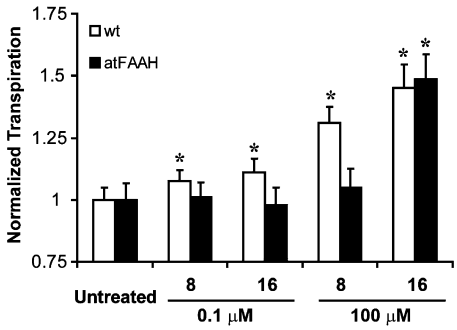

Figure 5. Effects of OdDHL (**8**) and L-homoserine (**16**) on transpiration in *A. thaliana* at 0.1 μM and 100 μM. Transpiration was evaluated in leaves of 7-day old *A. thaliana* (wt) or *A. thaliana* (Δ*AtFAAH*) seedlings (see Supporting Information Methods). Results are expressed as the mean of 5 leaves and normalized to untreated samples of either wt or Δ*AtFAAH A. thaliana* (± standard error). These results indicate that the exogenous addition of **8** and **16** increases the rate of transpiration in wt plants. However, the effect of OdDHL (**8**) on transpiration is attenuated in Δ*AtFAAH* plants, consistent with the hypothesis that the growth stimulatory effects of long-chain AHLs are dependent upon hydrolysis by *At*FAAH, yielding L-homoserine (**16**) as the active agent. Bars indicated with a * are statistically different ($p \leq 0.05$) relative to untreated controls.

or **16** at 100 μM caused a 40% greater weight loss relative to untreated control. However, in similar treatments of Δ*AtFAAH* plants, significant transpirative water loss occurred only in response to **16** but not to **8**. Water loss in samples exposed to 0.1 μM **8** or **16**, the concentration associated with increased root elongation, was reduced yet significant ($p \leq 0.05$; Figure 5), with a 10% increase in weight loss observed for both **8** and **16** in wild-type plants and again only for **16** in Δ*AtFAAH* plants. We note that transpirative water loss is an insensitive assay that likely underestimates the impact of AHL exposure on transpiration, and we currently are working to develop a more quantitative method to examine stomatal response. Nonetheless, these preliminary findings suggest that AHLs (after cleavage to L-homoserine (**16**)) can increase plant transpiration and provide additional support for the proposed model of FAAH mediated processing of AHLs in *A. thaliana*.

AHLs Stimulate Ethylene Production. While the transpiration assay data at 0.1 μM compound support the observed root growth enhancements in *A. thaliana* at low AHL concentrations, the transpiration assay data at 100 μM compound conflict with the observed deleterious growth effects at high AHL concentrations. Low to moderate exposures of the volatile phytohormone ethylene can inhibit stomatal closure in plants, potentially increasing transpiration;[50] however, we note that ethylene is also known to *inhibit* root elongation at elevated levels.[51] As L-homoserine (**16**) serves as a precursor in the biosynthesis of ethylene in plants (see Supporting Information Scheme 1 for a synopsis of this pathway), we hypothesized that AHL exposures could directly increase the concentration of this volatile phytohormone in *A. thaliana*. As such, low concentrations of AHLs would induce low levels of ethylene production, stimulating transpiration, and thus root growth. But at high AHL concentrations, the growth inhibitory effects of accumulating ethylene could supersede these increased transpiration effects, thereby generating the observed stunted phenotype. This model would support the observed progressive reduction in primary root length at AHL concentrations exceeding 1 μM.

In order to test this hypothesis, seedlings of wild-type *A. thaliana* were incubated with 1 μM aminoethoxyvinylglycine (AVG), a known ethylene biosynthesis inhibitor, as well as 100 μM OdDHL (**8**) or L-homoserine (**16**).[52] To account for increased plant growth due to the inhibition of native ethylene biosynthesis (by AVG), the root lengths of these seedlings were normalized to negative control plates containing 1 μM AVG only. As shown in Figure 6A, AVG treatments rescued seedlings from the growth inhibitory effects of both **8** and **16**, supporting the role of ethylene as a factor in the response of plants to elevated concentrations of AHLs. Similarly, the ethylene insensitive mutant of *M. truncatula*, Δ*skl*, displayed reduced sensitivity to the growth-inhibitory effects of both OdDHL (**8**) and L-homoserine (**16**) (see Supporting Information Figure 5)[53].

These findings were consistent with an increase in ethylene production as a major factor in AHL-induced growth inhibition at high concentrations. However, we sought to further examine the extent to which ethylene production was altered in response to AHL exposures. Methods exist for the detection of 1-aminocyclopropanecarboxylic acid (ACC), the direct ethylene biosynthetic precursor, which is a viable indicator for changes in ethylene levels in plants.[54] We evaluated the effect of OdDHL (**8**) (at 100 μM) on the level of ACC produced by wild-type *A. thaliana* seedlings using LC-MS/MS (see Methods). In brief, ACC was isolated from treated seedlings and derivatized with phenylisothiocyanate prior to LC-MS/MS analysis. The phenylisothiocyanate-derivatized ACC (PTC-ACC) yielded a collision induced dissociation peak of 136 m/z at 15.2 min (derived from the 237 m/z M+H parent ion) that was utilized to evaluate relative concentrations of ACC (Figure 6B). As seen in Figure 6C, the area of the 136 m/z fragment produced by seedlings treated with OdDHL (**8**) is roughly 2-fold greater than that observed in untreated controls, indicating that the levels of ACC (and presumably ethylene) are indeed increased in seedlings treated with OdDHL (**8**) at 100 μM.

In order to determine if the observed elevated ACC level could be derived from the *direct* incorporation of AHL-derived L-homoserine into ethylene biosynthesis, we synthesized a deuterated analogue of OdDHL (**18**; Figure 1B) and examined ACC isolated from wild-type *A. thaliana* seedlings treated with

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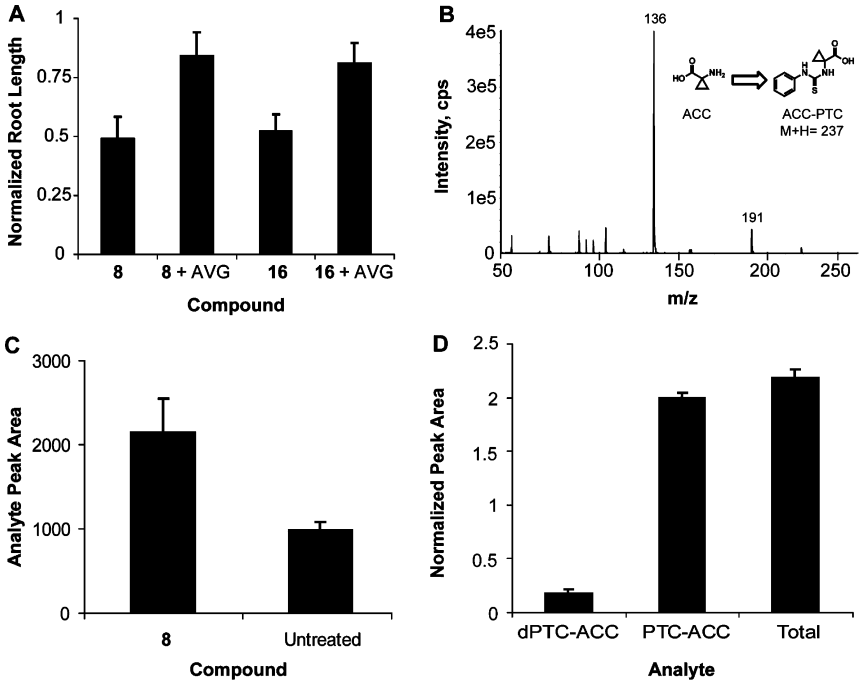

Figure 6. (A) Root length growth effects of 100 μM OdDHL (**8**) or L-homoserine (**16**) on 12-day old *A. thaliana* seedlings in the presence of AVG (1 μM). Data for **8** and **16** in the absence of AVG are shown normalized to the root length of untreated seedlings, and the AVG treated data are shown normalized to seedlings treated with 1 μM AVG only. Results shown are the average of 30 samples. (B) MS fragmentation pattern for PTC-ACC. Protonated PTC-ACC (237 m/z) yields CID fragments at 136 m/z and 191 m/z in a 10:1 ratio, a characteristic fragmentation pattern that was used to identify derivatized ACC in *A. thaliana* extracts (see Methods). (C) MS analyte peak area data for the 136 m/z CID peak obtained from extracts of treated (100 μM **8**) or untreated *A. thaliana* seedlings (average of three replicates). (D) MS analyte peak areas for derivatized ACC (PTC-ACC) and derivatized deuterated ACC (dPTC-ACC) extracted from *A. thaliana* seedlings treated with deuterated OdDHL (**18**) and normalized to untreated seedlings. Results shown are the average of three experiments.

18 (100 μM) for deuterium incorporation using LC-MS/MS. AHL **18** contains deuteriums at the 3- and 4-positions of the lactone ring; we predicted that three of these four deuteriums would be conserved in the biosynthetic processing of L-homoserine (**16**) to ACC (see Supporting Information Scheme 1). Thus, any ACC derived from exogenously added **18** would be distinguishable from other sources of ACC via LC-MS/MS due to an increase in m/z (240 for deuterated ACC vs 237 for ACC). As shown in Figure 6D, deuterated derivatized ACC (dPTC-ACC), while observable in *A. thaliana* extracts from plants treated with **18**, accounted for only a fraction ($\ll$10%) of the increased ACC (PTC-ACC) concentrations in response to **18**. Together, these results suggest that high concentrations of AHLs do stimulate ethylene production, but not through the direct incorporation of AHL-derived L-homoserine.

Can AHL-Induced Ethylene Responses Modulate Auxin Production? Crosstalk between the regulatory networks of the phytohormones ethylene and auxin is well established in *A. thaliana*.[51] However, there are conflicting reports as to whether auxin plays a role in the observed AHL-induced alteration of root system architecture in *A. thaliana*.[25,26] Prior studies have established changes in auxin homeostasis in white clover and *M. truncatula*, as well as the differential expression of auxin-responsive elements, upon exposure to AHLs.[27] As the results above implicate a role for ethylene in the response of plants to AHLs, we hypothesized that increased production of this volatile phytohormone could be modulating auxin homeostasis (including alterations in concentration, accumulation, and/or transport). To test for this possibility, we examined the effects of AHLs **5**–**8** and L-homoserine (**16**) in an *A. thaliana* auxin reporter strain (*DR5:GUS*) at 100 μM. This strain produces β-glucuronidase (GUS) as an indicator of auxin homeostasis. Increased GUS activity was observed in these reporter seedlings upon exposure to the four AHLs (Figure 7). As in our phenotypic growth assays, GUS activity was positively correlated with increasing AHL acyl chain length. Notably, comparable responses were observed for both OdDHL (**8**) and L-homoserine (**16**), consistent with a model in which AHL-derived L-homoserine

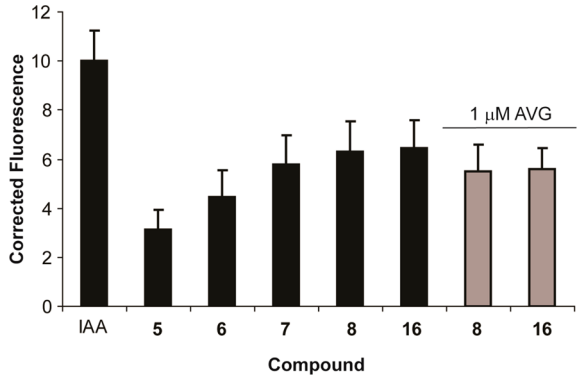

Figure 7. *A. thaliana* DR5:GUS auxin reporter data. *A. thaliana* DR5:GUS seedlings were treated with the indicated compound at 100 μM (black bars), or compound +1 μM AVG (gray bars), for 7 d. Auxin activity was assessed via the hydrolysis of MUG by β-glucuronidase, yielding a fluorescent product (see Methods). Fluorescence normalized to untreated samples and expressed relative to the fluorescence induced by 1 μM of a known auxin, indole acetic acid (IAA). Results shown are the average of three experiments.

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modulates auxin homeostasis (Figure 7). However, inhibition of ethylene biosynthesis (via addition of 1 μM AVG) did not have a significant effect on the abilities of **8** or **16** to activate the GUS reporter (Figure 7), indicating that auxin stimulation by **8** and **16** is not dependent on ethylene. Additional studies are needed to determine if the observed changes in auxin homeostasis upon AHL exposures stimulate ethylene production, or if the observed effects of AHLs on these two phytohormone pathways are independent, and are currently underway.

Summary and Conclusions. It has become increasingly clear that eukaryotes are sensitive to bacterial QS signals. However, the mechanisms by which the hosts perceive these molecules are largely unknown. Elucidating these mechanisms would not only broaden the fundamental understanding of interkingdom signaling but also could provide new routes to attenuate (or amplify) pathogenic and mutualistic associations. Much prior work in this area has involved the *ad hoc* testing of selected AHL signals on various aspects of plant growth and development. In the current study, we performed the first systematic analysis of the effects of a collection of native AHLs and byproducts on the model plants *A. thaliana* and *M. truncatula*. Our findings indicate that AHLs exert a concentration and acyl-chain length dependent effect on plant growth. AHLs with aliphatic acyl groups containing ≥12 carbons had the most pronounced effect, causing growth increases at submicromolar concentrations and growth inhibition at concentrations ≥50 μM. Unexpectedly, these growth effects were not dependent on the intact AHLs, but rather were caused by the AHL amidolysis product, L-homoserine (**16**). We present evidence that implicates the enzyme FAAH as an agent responsible for AHL hydrolysis in plants, thereby liberating **16**. The observed sensitivity of plants to AHLs with long aliphatic acyl groups is likely due to the preference of FAAH for NAE substrates that contain similar aliphatic groups.

Based upon our findings, we conclude that the observed AHL-induced growth effects in young plants (seedlings) can largely be attributed to L-homoserine (**16**). At low AHL concentrations, the presence of exogenous L-homoserine (**16**) increases plant transpiration, potentially increasing water and nutrient uptake as well as improving photosynthetic efficiency, all factors that can encourage plant growth. However, as the concentration of AHL increases (and thus L-homoserine (**16**)), ethylene also accumulates and the growth inhibitory effects of this volatile phytohormone ultimately exceed the benefits arising from the observed increase in transpiration. In contrast to the native alkyl HLs, the native aryl HL, *p*-coumaryl-HL (**13**) did not exert visible phenotypic effects in *A. thaliana*; this is may be due to the substrate specificity of *At*FAAH. Interestingly, we and others have identified a series of non-native aryl HL classes (many that mimic **13**) that are highly potent inhibitors and activators of QS in Gram-negative bacteria.[8−12] Our results for **13** suggest that such non-native AHLs could be utilized to study bacterial QS under native conditions in a host plant *without* initiating host responses. Ongoing studies in our laboratory suggest that this is indeed the case and will be reported in due course. These results also have implications for plant-associated bacteria that use aryl HLs as their native QS signals.

It is important to note that *At*FAAH expression levels are substantially lower (~15-fold) in the roots of mature *A. thaliana* relative to seedlings and the siliques (the seed capsule),[43,44] suggesting that FAAH-mediated AHL hydrolysis could be limited to specific stages of plant growth. The seedling growth phase has been the focus of the bulk of the studies to date on plant responses to AHLs,[25−27] likely due to the relative ease of experimental set up. However, it may well be the case that plants manifest differential responses to AHLs as a function of developmental stage, and this possibility needs to be considered in interpreting the results of such studies.

Overall, our results provide the first mechanistic insights into the perception of bacterial QS signals by plants and have implications on the current understanding of the role of QS signals at this interkingdom interface. It is likely that there are many players, in tandem with FAAH, that shape plant-bacteria interactions in response to QS signals. Nevertheless, we propose that by using the FAAH enzyme to process AHLs, plants may be able to "eavesdrop" on bacterial QS and directly impact the success of plant−bacteria associations. Such a model is shown schematically in Figure 8. As in QS, the result of this encounter is directly related to the available concentration of AHL and could confer physiological benefits upon both the plant and bacteria, depending on the concentration of the AHL. At low bacterial cell densities (and thus low AHL concentrations), the accumulation of L-homoserine (**16**) increases transpiration, encouraging water and nutrient uptake by the exposed roots. This mechanism may encourage the preferential colonization of the rhizosphere by QS bacteria.[55] In turn, high AHL concentrations may alert the plant to the presence of bacteria by stimulating defense responses such as increased ethylene production. Conspicuously, both AHL and L-homoserine (**16**) exposures have been associated with the

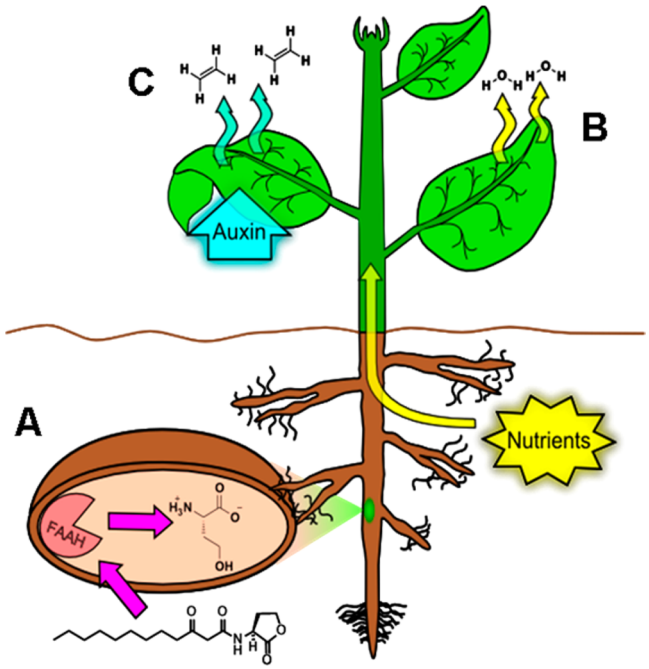

Figure 8. Schematic of a proposed model for plant responses to AHLs. (A) AHLs are taken up by the roots of plants and hydrolyzed by FAAH to yield L-homoserine (**16**). (B) The accumulation of L-homoserine (**16**) increases nutrient uptake via increased transpiration. This effect predominates at low concentrations of L-homoserine (**16**) and causes an increase in plant growth. (C) Accumulation of L-homoserine (**16**) stimulates both auxin and ethylene production. Accumulating ethylene at high concentrations of L-homoserine (**16**), inhibits plant growth.

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stimulation of defense responses in plants including, but not limited to, intracellular Ca^{2+}-spiking.[56,57] Of course, this proposed AHL-based strategy for detecting microbial populations would be unable to distinguish between pathogenic and potentially beneficial bacteria that utilize AHLs for QS. However, both beneficial and deleterious bacteria are initially treated as invaders by prospective host plants and must find strategies to avoid host immunity, consistent with a nonspecific detection strategy.[58] Indeed, one of the primary strategies utilized by plant-associated bacteria to avoid host defenses is through the disruption of specific elements of the ethylene response network.[58] Therefore, the ability of plants to distinguish between, and differentially respond to, two distinct AHL concentration regimes could play a role in directing the initial interactions between growing roots and populations of bacteria.

The possibility of extending this FAAH-based mechanism to other eukaryotes is exciting. Preliminary experiments in our laboratory show that recombinant human FAAH is capable of AHL hydrolysis, and analogous to *At*FAAH, has a preference for longer chain substrates and L-AHLs (see Supporting Information Figure 6). However, while many animals produce FAAH, L-homoserine (**16**) has no established role in these organisms, in contrast to plants. As such, FAAH-mediated responses to AHLs potentially could function as an interkingdom signaling strategy restricted to those eukaryotes in which L-homoserine serves a role in metabolism. The distribution of FAAH in animals (often in the brain and/or liver) also needs to be taken into account.[59] Further experiments are clearly needed to refine our understanding of the processing of AHLs by FAAH in plants and potentially in other eukaryotes and are currently underway.

METHODS

Materials, Strains, and Instrumentation. AHLs **1**−**4**, **7**, **9**−**13**, **14**, **15**, and **18** were synthesized according to previously reported methods.[8,9,11,60−62] AHLs **5**, **6**, and **8** and linoleoyl ethanolamide were purchased from Cayman Chemicals. Unless otherwise stated all other compounds (including L- and D-homoserine (**16** and **17**)), reagents, and media were purchased from Sigma-Aldrich and used according to enclosed instructions.

Seeds of wild-type (wt) *A. thaliana* (Columbia-0 ecotype) were purchased from Lehle Seeds. Seeds of *A. thaliana* Δ*AtFAAH* and the *E. coli* *At*FAAH-6xHis overexpression strain were provided by Elison Blancaflor (Noble Foundation).[43,44] Seeds of *A. thaliana* DR5:GUS (Col-0 ecotype) were provided by Patrick Masson (UW−Madison). Seeds of *M. truncatula* Jemalong A17 and Δ*skl* were utilized from our in-house seed stocks. Human recombinant FAAH was purchased from Cayman Chemicals.

Protein purification was performed on a GE ÄKTA-FPLC equipped with a GE HisTrap FF 5 column at 4 °C. LC-MS/MS data were obtained using an AB Sciex QTRAP 3200 triple quadrupole instrument. The interfaced HPLC was equipped with an Agilent 1100 series binary pump, a well plate autosampler, a column oven, and an Agilent Zorbax 1.8 μm StableBond C18 column (2.1 mm × 50 mm). HPLC solvents were 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Gradient elution was performed using the following solvent program: 2% B isocratic for 2 min, ramp to 12% B over 3 min, ramp to 30% B over 25 min, ramp to 60% B over 2 min, ramp to 98% B over 1 min, hold at 98% B for 1 min, then return to 2% B in 1 min and re-equilibrate for 7 min. The flow rate was 200 μL/min, and the column was maintained at 30 °C. Protein concentration was quantified using a Nanodrop 2000 UV/vis spectrophotometer (at 280 nm). Absorbance and fluorescence were measured using a Biotek Synergy 2 plate reader running Biotek Gen5 software (version 1.05).

Seed Germination and Growth. All seeds were germinated in the dark at room temperature (rt) after 48 h of imbibition at 4 °C. At 72 h postgermination, seedlings were transferred to plates of 0.5× Murashige-Skoog (pH: 6.0) (MS) medium (for *A. thaliana*) or modified Fahraeus medium (for *M. truncatula*) containing the desired concentration of compound.[63] For aminoethoxyvinylglycine (AVG) studies, growth media was additionally supplemented with 1 μM AVG. For hydroponic studies, germinated seedlings were transferred to sterile Erlenmeyer flasks containing 100 mL of liquid 0.5× MS medium.[64]

A. thaliana seedlings were grown for 10−14 d at rt with an 18:6 h day/night cycle (along with gentle shaking for hydroponic studies). *M. truncatula* seedlings were grown for 14 d at 22 °C with a 16:8 h day/night cycle. Seedling root length was measured at the end of the growth period with a ruler. Preliminary studies established that AHL exposures >200 μM inhibited *A. thaliana* germination and induced chlorosis by day 3 of growth. Additional phytotoxicity assays evaluating germination, total chlorophyll content, and ion leakage demonstrated that AHLs do not induce any appreciable toxicity in *A. thaliana* seedlings at concentrations <200 μM (Supporting Information Figures 2A−C; see Supporting Information for description of methods). Based on these results, we set the upper concentration limit for testing AHLs in our study to 100 μM to avoid toxicity.

Production and Purification of AtFAAH. The *E. coli* *At*FAAH-6xHis overexpression strain[65] was grown in Luria−Bertani (LB) medium supplemented with ampicillin (100 μg/mL). Cultures were grown to an OD_{600} of 0.6 at 37 °C (shaking at 250 rpm), after which *At*FAAH-6xHis production was stimulated by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM). Cultures were grown for 4 h at 37 °C (shaking at 250 rpm), and then centrifuged and flash frozen in liquid nitrogen. Prior to lysis, *E. coli* cells were thawed in IMAC A buffer (50 mM K_2HPO_4, 500 mM KCl, 20 mM imidazole, 5% glycerol, pH 7.9) at approximately 3 mL/g cells and incubated with lysozyme (3 mg mL^{-1} buffer) for 1 h. Cells were lysed by sonication (30 s, 3×; using a Fisher Scientific 550 Membrane Dismembrator sonicator), and the cell lysate was centrifuged at 40 000 rcf for 1 h. The resulting pellet was resuspended in IMAC A buffer supplemented with 20 mM *n*-dodecyl-β-D-maltoside (DDM) and allowed to shake overnight. The extracted pellet was then centrifuged at 75 000 rcf for 30 min to pellet cellular debris, and supernatant was injected onto an FPLC equipped with a HisTrap FF 5 mL column (pre-equilibrated with IMAC A supplemented with 0.2 mM DDM). The column was washed with IMAC A (containing 0.2 mM DDM), and protein was eluted using a 20−50 mM imidazole gradient. The entire *At*FAAH-6xHis purification process was carried out at 4 °C. The identity of *At*FAAH-6xHis was confirmed by SDS-PAGE and MALDI-TOF MS analysis (see Supporting Information Figure 4).

***At*FAAH AHL Hydrolysis Assay Protocol.** Purified *At*FAAH-6xHis was desalted and concentrated using a 10 kDa centrifugal filter into assay buffer (25 mM HEPES, 100 mM NaCl, 0.2 mM DDM, pH 8.0), and quantified using UV. Purification typically yielded 1 mg protein/L of cells. The activity of *At*FAAH-6xHis was confirmed by its ability to hydrolyze the natural substrate, linoleoyl ethanolamide, using the following protocol. *At*FAAH-6xHis (10 μg) and assay buffer were added to the wells of a 96-well multititer plate to give a volume of 190 μL per well. A 10-μL aliquot of the desired compound (dissolved in DMSO) was added to each well such that the compound concentration was 100 μM. [We note that assays monitoring the hydrolysis of radiolabeled substrates by FAAH are typically performed at comparable substrate concentrations.[43,44]] A positive control containing 100-μM linoleoyl ethanolamide and a negative control containing 10 μL DMSO only were also prepared. Plates were incubated for 30 min at 30 °C (with shaking at 150 rpm). The individual reactions were then filtered through 0.5 mL−10 kDa centrifugal filters. Aliquots of this flow-through (15 μL) were added to a fresh plate, and 90 μL of fluorescamine solution (1 mg mL^{-1} in acetone) and 195 μL of water were added. Fluorescence was immediately measured (390 nm excitation, 475 nm emission) using a plate reader. The ability of human FAAH to cleave AHLs was examined using this same protocol (Supporting Information Figure 6).

ACC Analysis by LC-MS/MS. *A. thaliana* seeds were germinated as described above and transferred to flasks containing 0.5× MS medium for hydroponic growth. Seedlings were grown for 4 d, at which time 100 μM OdDHL (**8**) was added to the growth medium, and the seedlings grown for an additional 10 d. Untreated seedlings were grown in parallel under the same conditions for use as negative controls. Seedling extract was prepared as previously described with slight modifications.[54] Briefly, 0.5 g of seedlings were frozen in liquid nitrogen, ground to a fine powder, and extracted for 1 h in 80% methanol at −20 °C. Cellular debris was pelleted at 75 000 rcf for 15 min, and the supernatant was cleaned via solid phase extraction, as follows. Supernatant was sequentially passed through a C18 SPE cartridge (Cayman Chemical), followed by a Resprep NH_2 WAX SPE cartridge (Restek). The resulting amino acid-rich flowthrough was diluted 1:1 with water, acidified using HCl to pH 2, and applied to a Grace SCX SPE column (Fisher Scientific). The column was washed extensively with 3 mM HCl and eluted with 5% NH_4OH. The resulting elutant was frozen and lyophilized. This dried material was derivatized for LC-MS/MS analysis using phenylisothiocyanate (PITC) (treatment with solution of 3 mL 10:5:3:2:1 ACN/pyridine/water/TEA:PITC, 1 h). Derivatized samples were washed with 10:1 heptane/ethyl acetate (2×), followed by 2:1 heptane/ethyl acetate (2×). The aqueous layer was isolated, lyophilized, resuspended in water, and submitted for LC-MS/MS analysis for PTC-ACC.

MS was performed during HPLC elution using a multiple reaction monitoring (MRM) approach. Fragment ions produced by PTC-ACC were determined by direct infusion. Protonated PTC-ACC was detected at *m/z* 237 and produced collision induced dissociation (CID) fragments at *m/z* 136 and 191 at a collision energy (CE) setting of 25. During HPLC elution, *m/z* 136 and 191 were detected as fragments of precursor *m/z* 237 continuously with 100 ms dwell times at each precursor-to-product transition (*m/z* 237 to 136 and *m/z* 237 to 191) at 15.2 min. PTC-ACC detection was validated by comparing HPLC retention time with a PTC-ACC standard. Data analysis was performed using AB Sciex Analyst 1.4 software.

Detection of Deuterated ACC Derived from Deuterated OdDHL (18) via LC-MS/MS. Wild-type *A. thaliana* was germinated as described above and grown for 4 d hydroponically, after which compound **18** was added to the growth medium (to make a 50 μM solution), and seedlings were grown for an additional 7 d. A second dose of **18** (to make 50 μM) was added on day 11, after which the seedlings were allowed to grow for a final 3 d (14 d total experiment). *A. thaliana* seedling extract was prepared and analyzed by LC-MS/MS as described above. During HPLC elution, *m/z* 136 and 194 were detected as fragments of precursor *m/z* 240 at 15.1 min, and were used to identify deuterated PTC-ACC in *A. thaliana* extract.

***A. thaliana* Auxin Reporter Assay Protocol.** *A. thaliana* DR5:GUS reporter lines were grown using the same test tube method as described for the transpiration assay (see Supporting Information). After 7 d, the media was exchanged for fresh MS media containing the compound of interest. After 48 h of compound exposure, seedlings were homogenized, and GUS activity was evaluated using the fluorescent substrate 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as previously described.[66] Fluorescence was evaluated 30 min after the addition of 4-MUG (360 nm excitation, 460 nm emission) using a plate reader.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Email: blackwell@chem.wisc.edu.

Present Addresses

[‖]Department of Biological Sciences, 150 W. University Boulevard, Florida Institute of Technology, Melbourne, Florida 32901, United States

[⊥]Department of Biology, 201 Donaghey Avenue, University of Central Arkansas, Conway, Arkansas 72035, United States

Author Contributions

[§]A.G.P. and A.C.S. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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