Selection of appropriate autoinducer analogs for the modulation of quorum sensing at the host-bacteria interface

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ABSTRACT: Bacteria regulate a variety of phenotypes in response to their population density using quorum sensing (QS). This phenomenon is regulated by small molecule or peptide signals, the best characterized of which are the *N*-acyl L-homoserine lactones (AHLs) utilized by Gram-negative bacteria. As many QS-controlled phenotypes, notably pathogenicity and symbiosis, can profoundly impact host eukaryotes, there is significant interest in developing methods to modulate QS signaling and either ameliorate or augment these phenotypes. One strategy has been the use of non-native AHL analogues to agonize or antagonize specific AHL receptors. This approach is complicated, however, by the potential for prospective hosts to respond to both native AHLs as well as synthetic analogues. Accordingly, identifying AHL analogues with little or no activity towards eukaryotes is important in developing QS modulation as a strategy for the regulation of prokaryotic behaviors. Herein, we utilize the model plant *Arabidopsis thaliana* to characterize eukaryotic responses to a variety of synthetic AHL analogues to identify structural elements of existing scaffolds that may elicit responses in prospective hosts. Our results indicate that, while many of these compounds have no discernable effect on *A. thaliana*, some elicit strong phenotypes similar to those produced by auxin, a hormone involved in almost all aspects of plant development. We outline concentrations and chemical scaffolds ideal for deployment on plant hosts for the regulation of QS. This approach should be exportable to other eukaryotes for the selection of optimal AHL tools for the study of QS at the host-microbe interface.

INTRODUCTION

Many common bacteria manifest collective behaviors based on population density in a phenomenon known as quorum sensing (QS) [1,2]. QS is mediated by small molecule or peptide signals (generically classified as autoinducers) and their cognate receptor proteins [3,4]. The concentration of autoinducer increases with cell density, and once the concentration reaches a threshold level, productive autoinducer:receptor binding occurs that subsequently regulates gene transcription. QS genes coordinate a broad variety of phenotypes such as biofilm and virulence factor production, nodulation, conjugation, swarming, and bioluminescence [5,6]. Many of these phenotypes are only beneficial to these microbes at high cell densities. For example, by coupling virulence factor production to population density, the opportunistic pathogen *Pseudomonas aeruginosa* can evade host defenses until sufficient quantities of bacteria are present to survive the host immune response [7].

Due to the often dramatic impact of QS phenotypes on prospective host eukaryotes, there is considerable interest in developing strategies to modulate QS signaling pathways [8–11]. Small molecule-based approaches, utilizing synthetic autoinducer analogs to agonize or antagonize QS receptors, have been successful in this regard for the attenuation of specific phenotypes and for further delineation of QS mechanisms [10,12–19]. Much of this past work has focused on the *N*-acyl L-homoserine lactone (AHL) signals and their cognate LuxR-type receptors utilized by Gram-negative bacteria [2]. AHLs are characterized by a conserved L-homoserine lactone "head" group, with receptor specificity conferred by variations in the attached acyl "tail" (Figure 1) [8]. Common variations include acyl tail length (4–18 carbons), the presence or absence of a *cis*-alkene, and the oxidation state at the 3-position. Four AHLs have also been identified that are not derived from common fatty acids: *p*-coumaryl, cinnamoyl, and phenylacetyl homoserine lactones (pcHSL, cHSL, and PA-HSL, respectively), and a branched derivative, isovaleryl homoserine lactone [20–23].

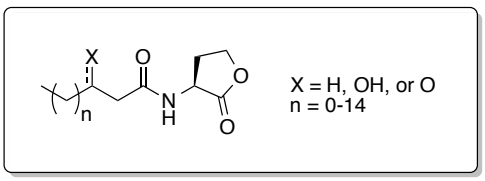

Figure 1: Generic structure of the AHL-type QS signals used by Gram-negative bacteria.

The screening of AHL-inspired compound libraries in cell-based assays that report LuxR-type receptor activity has identified several chemical scaffolds with the potential to strongly agonize or antagonize QS circuits in bacteria, including *P. aeruginosa* [24–30], *Agrobacterium tumefaciens* [12,13], *Vibrio fischeri* [31], *Acinetobacter baumannii* [32], *Pectobacterium carotovorum* [17,33], *Sinorhizobium meliloti* [34], and *Chromobacterium violaceum* [35]. The utility of synthetic AHL analogues (SAHLAs) as modulators of QS under native conditions (i.e., whilst the bacteria colonizes its natural host) has also been validated in several systems, including: (i) inhibiting virulence factor production by *P. carotovorum* in infections of *Solanum tuberosum* (potato) [33], (ii) enhancing QS-mediated nodulation between strains of *Sinorhizobium meliloti* and its legume host *Medicago truncatula* [34], and (iii) altering *V. fischeri* bioluminescence in the light organ of *Euprymna scolopes*, the bobtail squid [36]. The success of these trials is establishing both the concentration and dosing times required for effectively regulating QS signaling *in situ*.

However, one critical feature of these studies of QS at the host-microbe interface that requires further attention is delineating the sensitivity of the eukaryotic host to both native AHLs and SAHLAs. For example, the *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) signal utilized by *P. aeruginosa* can modulate growth and phytohormone production in plants [37–39]. In addition, OdDHL and other long chain AHLs can influence the total number of nodules that form on the roots of *M. truncatula* during *S. meliloti* colonization [34]. Other AHL-induced responses in eukaryotic systems have been reported [40,41], however, the mechanisms by which AHLs elicit these responses remains unclear, and are the target of ongoing studies. Nevertheless, these effects in the host complicate the study of LuxR-type QS outcomes at the host-microbe interface with SAHLAs (or other chemical scaffolds). Such approaches hinge on the identification of compounds with potent agonist or antagonist activity towards LuxR-type circuits but with little or no interfering activity in eukaryotes. Defining such scaffolds was a broad motivation for the current study.

We previously utilized the model plant *Arabidopsis thaliana* as a system for evaluating eukaryotic responses to native AHLs due to its sensitivity to these signals, the array of mutants and reporters that are readily available, and its rapid and well-defined growth. These past studies, as well as that of others [34,38,39], have provided several metrics for evaluating AHL responses in *A. thaliana* seedlings, including root elongation and changes in the regulation of the important phytohormones auxin and ethylene. We sought to build and expand on these prior studies to test the impact of SAHLAs on plant growth and development. Our ultimate goal was to identify scaffolds that do and do not elicit effects on the host.

Herein, we report the results of phenotypic assays, phytohormone reporter assays, and mass spectrometry experiments to evaluate the responses of *A. thaliana* to some of the most potent SAHLA-based LuxR-type receptor agonists and antagonists reported to date. Our results indicate that while certain classes of SAHLAs have limited effects on the plant host, other synthetic scaffolds, (e.g., certain phenylacetyl HLs (PHLs)) can have significant effects that are caused, at least in part, by interfering with endogenous hormone pathways in *A. thaliana*. Namely, specific members of this class were observed to directly modulate a receptor for the plant hormone auxin (TIR1) that plays a crucial role in cell elongation and plant growth [42,43]. Based on these findings, we discuss structural features in SAHLAs that should be prioritized, as well as the optimal compound concentration range to be utilized, to limit host responses and facilitate the use of SAHLAs as regulators of QS at the host-microbe interface.

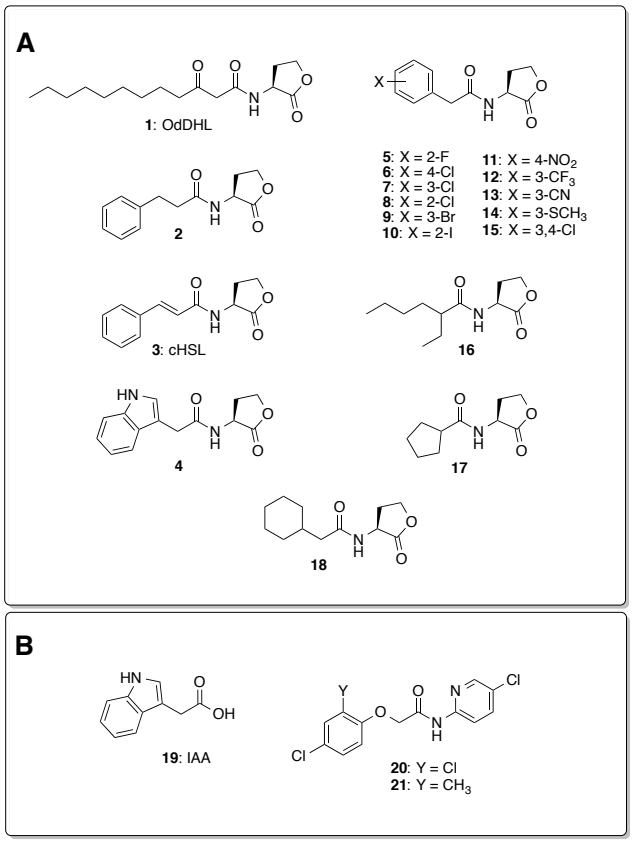

Figure 2: Compounds examined in this study. (A) Structures of natural and synthetic AHL analogues (SAHLAs). Original identifiers for AHLs from source papers included in parentheses [13,31,44–46]: **1** (OdDHL), **2** (B9), **3** (B10), **4** (B13), **5** (C4), **6** (C5), **7** (C6), **8** (C7), **9** (C8), **10** (C12), **11** (C13), **12** (E3), **13** (E5), **14** (E7), **15** (E11), **16** (S1), **17** (S4), and **18** (S6). (B) Structures of the natural auxin **19**, (indole acetic acid (IAA)) and synthetic auxins **20** (AA1) and **21** (AA2).

RESULTS AND DISCUSSION

SAHLAs can alter *A. thaliana* seedling growth. Root morphology, specifically primary root length, has been used previously as a straightforward phenotypic marker for sensitivity to low molecular weight compounds, including AHLs, in both *A. thaliana* and *M. truncatula* [38,39,47]. We initiated our study using this assay in *A. thaliana* to measure responses to a representative set of seven SAHLAs identified by our laboratory that act as agonists and antagonists of LuxR-type receptors in a range of bac-

teria [13,31,33,44]. This initial set consisted of phenylpropionyl HL **2**, the related (and naturally occurring) cinnamoyl HL (cHSL) **3**, indole containing-HL **4**, and the substituted phenylacetyl HLs (PHLs) **5**, **7**, **9**, and **10** (Figure 2A). The native AHL from *P. aeruginosa*, OdDHL (**1**), served as a control as the effects of this molecule on plants has been well characterized [34,38,39].

A. thaliana seedlings were grown on solid media containing these compounds at either 0.1 μM or 50 μM (see Methods), concentrations at which naturally-occurring AHLs have been shown to either induce or inhibit root elongation, respectively [34,39]. Exposures to **1** (OdDHL) increased root elongation at 0.1 μM and inhibited growth at 50 μM, consistent with previous studies (Figure 3) [39]. In contrast, none of the tested compounds altered primary root length at 0.1 μM, and only SAHLAs **4**, **7**, **9**, and **10** significantly inhibited primary root length at 50 μM (Figure 3). Normal growth could be restored in these seedlings by transferring them to SAHLA-free plates, and no obvious disruption of membrane integrity was observed at 50 μM (based on electrolyte-leakage assays [39]; see Supplementary Figure 1), arguing against any toxic effects from these compounds. For all four of these SAHLAs, inhibition of primary root length coincided with an increase in both lateral root number as well as root hair density. Representative examples of these effects on *A. thaliana* with indole HL **4** and 3-bromo PHL **9**, and the lack thereof for phenylpropionyl HL **2** and 2-fluoro PHL **5**, can be seen in Figure 4. These effects on *A. thaliana* seedlings are consistent with the exogenous addition of an auxin-type small molecule, an important class of phytohormones in plant growth and development (Figure 2B)[48]. Indeed, exogenous addition of the auxin indole acetic acid (IAA (**19**), Figure 2B) replicated the growth effects of **4** and **9** (Figure 4).

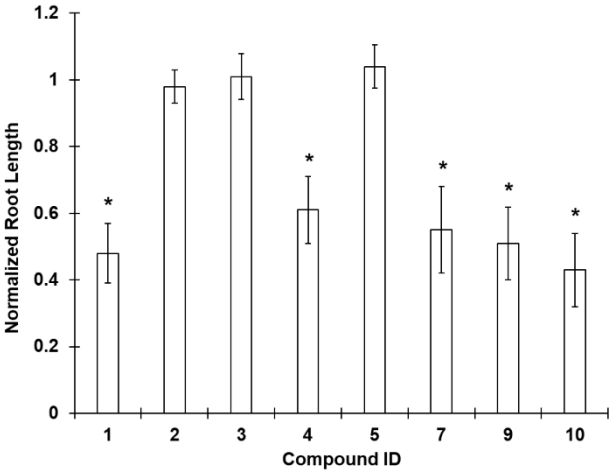

Figure 3: Effect of AHLs on *A. thaliana* root length. Two day-old seedlings of *A. thaliana* were transferred to plates containing 50 μM of the indicated compound. Results are the mean growth of 30 seedlings, normalized to untreated (compound free) controls after 12 days. '*' indicates samples statistically different from untreated controls ($p<0.05$). Compound **1** = OdDHL.

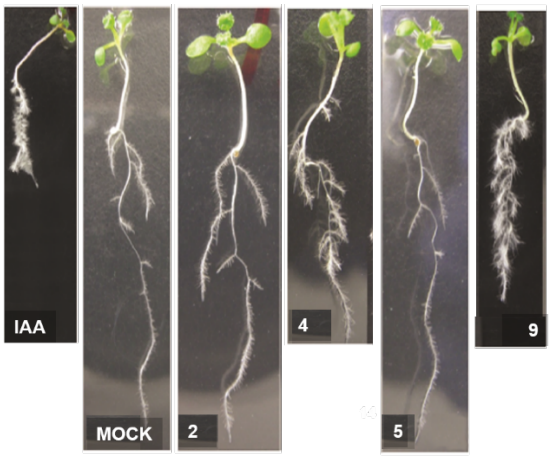

Figure 4: Effects of small molecules on *A. thaliana*. Seedlings of *A. thaliana* were grown on the indicated compound at 50 μM. Images were collected after seven days. MOCK = sample treated with an equal volume of DMSO, the carrier solvent for the tested molecules. IAA = indole acetic acid (**19**).

SAHLAs activate an auxin reporter in *A. thaliana*. The results of the root growth assays suggested that auxin-associated pathways could be involved in the observed SAHLA effects on *A. thaliana* seedlings. To test this hypothesis, we utilized a DR5:GUS reporter line of *A. thaliana* that produces the enzyme β-glucoronidase (GUS) in response to auxin (see Methods) [49]. Treatments of this *A. thaliana* reporter with SAHLAs **4**, **7**, **9**, and **10** at 50 μM showed a substantial increase in GUS expression (60–90% relative to IAA (**19**) control) after 6 hours (Figure 5). Exposure to 50 μM OdDHL (**1**), included as a positive control, also induced GUS expression by 50% (relative to IAA (**19**)), consistent with our prior studies [39]. In contrast, the SAHLAs that had no impact on root growth (i.e., **2**, **3**, and **5**) also had minimal effect on GUS activity. These results are consistent with SAHLAs **4**, **7**, **9**, and **10**, as well as OdDHL (**1**), activating auxin signaling in *A. thaliana*.

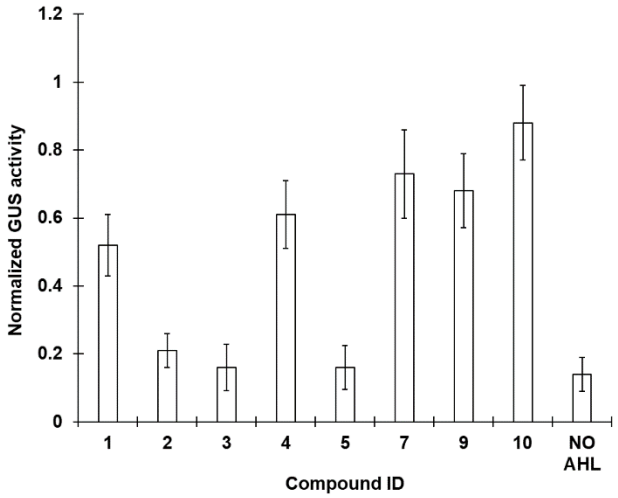

Figure 5: Effects of AHLs on GUS expression in an *A. thaliana* DH5::GUS reporter line. Five day-old seedlings of *A. thaliana* were transferred to liquid cultures with 50 μM of the indicated compound. After incubation for 48 h, 15 seedlings were harvested and evaluated for GUS activity using the 4-MUG fluorescent substrate. Results are

expressed relative to IAA (**19**) control (1 μM; set to 1), as the average of five trials. Compound **1** = OdDHL.

Select SAHLAs can directly activate an auxin receptor. Auxins are a class of structurally related compounds that include IAA (**19**), phenylacetic acid (PAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). All of these compounds are aryl carboxylic acids [48]. This architecture has been shown to be critical for binding interactions with their native receptor in plants, TIR1 [42,50]. Conspicuously, these molecules share structural similarities to the PHL class of SAHLAs (Figure 2) and to the naturally occurring, unsubstituted PHL signal, PA-HSL[23]. Indole HL **4** is also structurally similar to IAA. Based on the observed effects of the indole HL **4**, as well as the PHLs **7**, **9**, and **10** on *A. thaliana* seedling root elongation and GUS production in the DR5 reporter line, we hypothesized that these compounds may function in plants by directly binding the TIR1 auxin receptor.

TIR1 binding of IAA, or other auxin-like compounds, is known to enhance TIR1's association with IAA7, a transcriptional repressor protein, targeting the latter for degradation. The degradation of IAA7 then relieves repression of auxin-associated genes [42,51]. We adapted a high-throughput TIR1/IAA7 yeast two hybrid (Y2H) assay to screen our compounds for their ability to interact with TIR1 by stabilizing the TIR1/IAA_7 complex in *Saccharomyces cerevisiae* (yeast) [52]. In this system, the exogenous addition of auxins (or synthetic analogues) drives the association of TIR1 and IAA7 proteins, activating β-galactosidase (β-gal) production. The TIR/IAA7 yeast reporter was incubated with 100 μM OdDHL (**1**), cHSL (**3**), an expanded set of SAHLAs (**2**, **4**–**18**), and two weak synthetic auxin analogues AA1 (**20**) and AA2 (**21**) [45,53] (Figure 2). Notably, this set of SAHLAs contained 11 PHLs to both further probe this important structural class of LuxR-type receptor modulators in plants and examine their structural similarity to auxins. Compounds were considered auxin agonists in the Y2H assay if they were able to induce β-gal activity to ≥30% relative to the IAA (**19**) control (at 1 μM). We observed that the SAHLAs that displayed auxin-like activity in *A. thaliana* seedlings (**4**, **7**, **9**, and **10**; Figures 3–5), along with two additional PHLs (**13** and **14**), induced β-gal production by >40% (Figure 6). Agonistic activity was also observed for the auxin controls AA1 (**20**) and AA2 (**21**), as expected. Except for 2-iodo PHL **10**, all of the PHLs active in the Y2H assay had substituents in the 3-position of the aryl ring. Interestingly, OdDHL (**1**) did not display significant activity in this reporter assay, suggesting that this native AHL does not associate with the TIR1 auxin receptor.

Next, we evaluated the ability of the Y2H-TIR1 assay to predict SAHLA scaffolds that induce auxin phenotypes in *A. thaliana* seedlings. We selected PHLs **11** and **14** for testing, as they exhibited low and high activity in the Y2H assay, respectively. We reasoned that the 4-NO_2 PHL **11** would not have significant effects on growth, while 3-SCH_3 PHL **14** would induce significant growth effects. This hypothesis was supported, as no abnormal phenotypic characteristics were observed upon treatment of *A. thaliana* seedlings with **11**, while **14** showed reduced root elongation and increased lateral root number, consistent with the auxin phenotype (Figure 6, inset pictures).

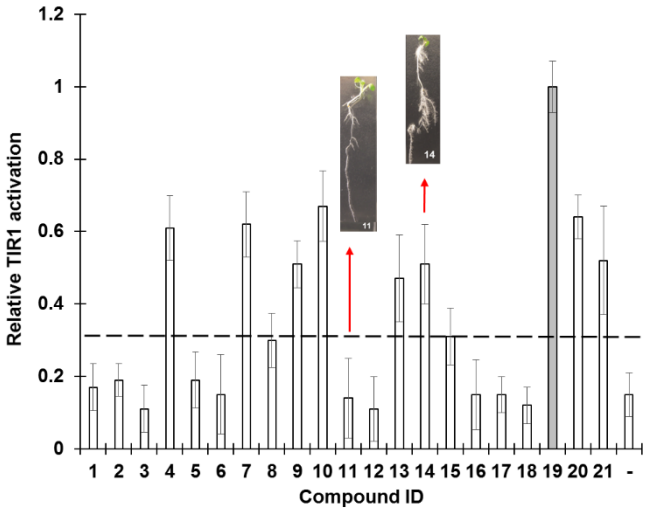

Figure 6: TIR1/IAA_7 activation by synthetic compounds as detected using a yeast two-hybrid (Y2H) assay. Results are expressed relative to 1 μM IAA (**19**, shown in grey) controls, as the average of five trials. The dashed line indicates 30% of IAA activity, which was used as a minimum threshold. '-' indicates AHL-free control sample. Compounds **11** and **14** were evaluated for phenotypic effects in *A. thaliana* seedlings as described; representative growth assay images are shown as insets.

SAHLAs can be hydrolyzed by *A. thaliana* to auxin-like products. Our prior studies of plant sensitivity to AHLs demonstrated that long chain native AHLs (e.g., OdDHL, **1**) and their amide hydrolysis product, L-homoserine, had comparable effects on root growth, implicating an amidase in their processing/perception pathway [39]. Both compounds were capable of limiting root elongation, yet unlike the observed SAHLA effects, they did not increase root hair density or lateral root number. Through a series of knockdown, overexpression, and *in vitro* studies, we established the *A. thaliana* fatty acid amide hydrolase (AtFAAH) as a primary candidate for cleavage of aliphatic AHLs. Purified AtFAAH was unable to cleave the amide bond in aryl HLs, however, including the PHLs [39]. That said, *A. thaliana* possesses at least seven enzymes with high homology to known amidases, including AMI1, which catalyzes the conversion of indole-3-acetamide into IAA (**19**) [54,55]. We hypothesized that one of these other amidases in *A. thaliana* could be capable of cleaving aryl HLs, thereby generating L-homoserine and the corresponding carboxylic acid. These byproducts could then explain the observed auxin-like effects for the SAHLAs tested in this study. Specifically, in the case of indole HL **4** and PHLs, the carboxylic acid could then function as an auxin analogue, while the L-homoserine moiety could reduce root length via indirect modulation of auxin signaling, as we observed previously [39].

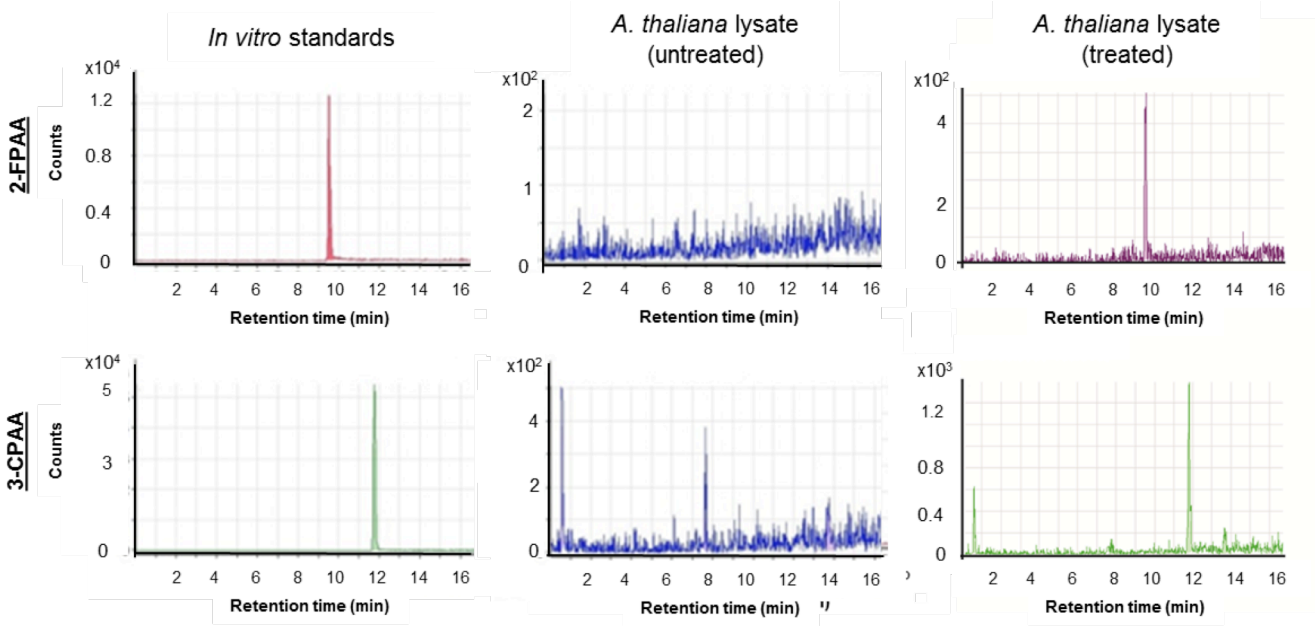

Figure 7: LC-MS analysis of SAHLA hydrolysis products produced upon exposure to *A. thaliana*. Extracted ion chromatograms (EICs) for 2-FPAA and 3-CPAA ions in: (*left*) standard samples of 2-FPAA (top) and 3-CPAA (bottom); (*middle*) lysates of untreated control plants; and (*right*) lysates of PHL **5** (top) and PHL **7** (bottom) treated plants.

To probe if SAHLAs can be cleaved *in planta*, we selected two PHLs for further study (**5** and **7**). The 2-fluoro PHL **5** had no significant effects on growth, GUS production, or β-gal production (in the Y2H assay), while seedlings treated with the 3-chloro PHL **7** displayed an auxin-like phenotype and significant increases in both GUS and β-gal production. Seedling growth assays with 50 μM 2-fluorophenylacetic acid (2-FPAA), the carboxylic acid derived from PHL **5**, had no significant effects on *A. thaliana* root system architecture. In contrast, 3-chlorophenylacetic acid (3-CPAA), the carboxylic acid derived from PHL **7**, induced an auxin-like phenotype at 50 μM similar to the parent PHL (see Supplementary Figure 2). These results suggested that cleavage of the amide in PHL **7** by *A. thaliana* could generate a carboxylic acid with auxin-like effects.

We further investigated the ability of *A. thaliana* to cleave PHLs **5** and **7** using liquid chromatography-mass spectrometry (LC-MS). The carboxylic acid products that would be formed by amidase-mediated cleavage (2-FPAA and 3-CPAA) have approximate retention times of 10 and 12 minutes, respectively (Figure 7, left panels). After exposure to 50 μM **5** or **7** for 10 days, *A. thaliana* seedlings were homogenized, subjected to extraction with both aqueous and organic solvents, and the extracts were analyzed by LC-MS. Neither of the PHL-derived acids (2-FPAA or 3-CPAA) was present in the chromatograms of untreated *A. thaliana* seedlings (Figure 7, middle panels). However, the chromatograms for lysates of treated *A. thaliana* seedlings revealed the presence of their respective carboxylic acids (Figure 7, right panels), suggesting that cleavage of the amides in these PHLs does occur in plants. No significant hydrolysis of the amide bond in either **5** or **7** was observed in control samples stored over the 10 days of the assay in the absence of plants (as would be expected for amides), indicating that cleavage was due to the presence of *A. thaliana*.

Normalization of LC peak areas to an internal standard (see Methods) indicated that treatment of *A. thaliana* with 100 μM of either **5** or **7** resulted in the accumulation of 2.38 and 1.92 nmol/g of carboxylic acid, respectively. Naturally occurring concentrations of IAA (**19**), as determined by MS, were typically < 1 nmol/g of fresh weight. While these concentrations are comparable, it is important to note that unlike IAA, which is well-established to be biologically active at nanomolar concentrations, both fragments derived from the cleavage of **7**, 3-CPAA and L-homoserine, are only active at micromolar concentrations,[39] arguing against them being a source of the observed auxin-like activity. Furthermore, based on the volume of the plate (15 mL), the starting SAHLA concentration (100 μM), and the molecular weight of the products, the accumulated amount of cleavage products represents < 1% of the starting material. We therefore propose that the observed activities of SAHLAs in *A. thaliana* seedlings, such as the PHLs, are likely due to the intact structures as opposed to their degradation products.

Summary and outlook. The application of synthetic small molecules to modulate and probe bacterial QS in the presence of eukaryotic hosts provides an attractive strategy to study the role of this signaling mechanism in the regulation of bacterial populations in their native environments. The viability of this strategy depends, at least in part, upon the development of ligands to which prospective eukaryotic hosts have limited sensitivity, and thus are bacterial selective. In the current study, we utilized the model plant *A. thaliana* to examine if eukaryotes

were also sensitive to certain synthetic AHL analogues, i.e., SAHLAs, many of which we have demonstrated to be highly potent chemical modulators of LuxR-type QS.

We combined phenotypic and phytohormone assays to evaluate a number of SAHLAs for their effects on *A. thaliana* seedlings. We discovered that some of our analogues (particularly the PHLs) elicit strong auxin-type phenotypes in *A. thaliana*. Results of a Y2H screen strongly suggest that this response is a consequence of their direct interactions with the TIR1/IAA_7 co-receptor. These experiments indicate that substituted PHLs (more specifically, those with *meta*-aryl substitutions within the preliminary set of compounds tested herein) and AHLs with acyl tails similar to known auxin molecules (i.e., indole HL **4**) are unwise choices as scaffolds to modulate QS, at least on plant hosts, due to their ability to act as auxins in *A. thaliana*. Furthermore, mammals possess TIR1 F-box homologues that associate with Skp1 and CDC53 proteins to assemble ubiquitin ligase complexes, suggesting that SAHLAs may have unwanted activities among eukaryotes other than plants [56]. Accordingly, the results of this study have implications for the application of SAHLAs in a broader range of host-microbe experiments.

We previously demonstrated that the activity of naturally occurring AHLs in plant hosts was dependent upon cleavage by the enzyme FAAH to yield L-homoserine, the active compound. In the current study, however, we found that an insufficient amount of either carboxylic acid or L-homoserine derived from substituted PHLs (a lead SAHLA scaffold) accumulates to function as a signal. This is a positive result since it suggests that secondary effects of SAHLA degradation may be a low priority in the design of synthetic QS modulators. However, in view of the small set of synthetic modulators studied so far in plants, and in eukaryotes in general, SAHLA degradation may need to be evaluated on a scaffold-by-scaffold basis during probe development in the future.

Finally, while our results highlight SAHLA scaffolds that have the potential to interact with host eukaryotes, it is important to note that the EC_{50} and IC_{50} values for many of these compounds in LuxR-type receptors range from low nanomolar to low micromolar concentrations [13,34]. Meanwhile, deleterious effects on growth, auxin, and ethylene pathways only begin at 10 μM [39], suggesting the possibility of a dosing range for synthetic analogues within which these effects can be avoided. Perhaps more importantly, many of the SAHLAs tested herein *do not appear* to have any significant auxin activity and these growth effects then become a non-issue. This set includes compounds (i.e., **2**, **3**, **5**, **6**, **11**, **12**, and **16–18**; Figure 2A) that we have already shown to be potent QS regulators. For example, compounds **2**, **3**, and **5** are antagonists of LuxR in *V. fischeri* and ExpR2 in *P. carotovorum* [17]. Similarly, **6**, **11**, and **12** are antagonists for both of these receptors as well as TraR in *A. tumefaciens* [13]. Branched SAHLAs, represented by **16**, are strong agonists of QscR in *P. aeruginosa* [15]. While alternative modes of activity other than the auxin pathway cannot be discounted, the findings reported here support the utility of synthetic QS modulators in manipulating microbial populations on a plant host in the absence of major effects on the host, and constitute a step forward in developing this chemical approach.

METHODS

Materials. Wild-type Arabidopsis seeds (Columbia-0 ecotype) were purchased from Lehle seeds (Rock Round, TX). *DR5::GUS* seedlings of *A. thaliana* were provided by P. Masson (UW–Madison). The TIR1/IAA7 yeast two hybrid (Y2H) system was provided by M. Estelle (UC San Diego). Native and non-native AHLs were utilized from pre-existing stocks that were synthesized according to published protocols [13,31,44,46]. Synthetic auxins **20** and **21** were prepared using standard amide coupling protocols and yielded NMR and MS characterization data as expected [53]. Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Plant growth assays with synthetic AHLs. *A. thaliana* WT seeds were surface sterilized in 70% ethanol for 2 min, rinsed 5X with sterile water, and plated onto 0.5X Murashige and Skoog (MS) medium (Caisson Labs, Logan, UT) containing 3% sucrose and 0.4% gelzan (pH: 5.7). Seeds were stored at 4 °C for 48 h, and then germinated in the dark at room temperature. Seventy-two h post-germination, seeds were transferred to MS plates containing the desired concentration of AHL and were grown for 7 d at room temperature with a 16:8 hour day/night cycle.

DR5::GUS assays. Seedlings of *A. thaliana* DR5::GUS were treated with 50 μM of the indicated compound for 48 h, after which they were homogenized and evaluated for GUS activity using the fluorescent substrate 4-methylumbelliferyl-β-D-glucuronide (4-MUG) [39]. Fluorescence was evaluated 30 min after the addition of 4-MUG (360 nm excitation, 460 nm emission) using a plate reader (SpectraMax i5 running SoftMax Pro software [version 7])

Yeast two hybrid screens. The *S. cerevisiae* TIR1/IAA7 strain was maintained at 4 °C on plates containing glucose synthetic dropout (SD) medium (6.7 g/L yeast nitrogen base [BD Biosciences, Sparks, MD], 0.7 g/L -His/-Trp/-Ura amino acid dropout mix [Clontech, Mountain View, CA], 2% glucose, 2% agar, pH: 5.8). Overnight cultures (30 mL) were made by selecting a single yeast colony and inoculating into liquid glucose SD medium. Cultures were grown for 24 h at 30 °C (with shaking at 250 rpm) to an optical density at 600 nm (OD_{600}) of 2.0.

Cultures were centrifuged at 1700 relative centrifugal force (rcf), and pellets washed with galactose/raffinose SD medium (6.7 g/L yeast nitrogen base, 0.7 g/L -His/-Trp/-Ura amino acid dropout mix, 2% galactose, 1% raffinose, pH: 5.8). Washed pellets were diluted 1:10 in fresh galactose/raffinose SD medium, and yeast were added to the wells of a 96-well plate containing AHL (or IAA for positive control) such that the final ligand concentration was 100 μM. Plates were wrapped in aluminum foil and incubated for 24 h at 30 °C (with shaking at 250 rpm).

To assay for β-galactosidase production, yeast were pelleted and re-suspended in yeast lysis buffer (Z-buffer), containing 4 lytic units of zymolyase (Zymo Research, Irvine, CA) per well. Plates were allowed to incubate at 37 °C for 1 h. Cells were then lysed with SDS/chloroform, and β-galactosidase production assayed by adding the substrate chlorophenol red-β-D-galactopyranoside (CPRG, Roche) and measuring absorbance at 580 nm using a plate reader.

Extraction and LC-MS analysis to assess AHL hydrolysis by *A. thaliana*. For plant analyses, *A. thaliana* WT seeds were surface sterilized and germinated on MS media as described above. Germinated seedlings were transferred to liquid MS medium containing 100 μM of AHL **5** or **7**. Seedlings were allowed to grow for 10 d hydroponically, dosing in an additional 50 μM AHL at day 5 of growth. Seedlings were rinsed thoroughly and cells were lysed using liquid nitrogen and mechanical grinding. A 0.5 g portion of tissue was extracted with 5 mL of a 2-propanol/H_2O/HCl (2:1:0.002 v/v/v) mixture at 4 °C for 30 min after the addition of 1 nmol of 3-bromophenylacetic acid (3BPAA) as an internal standard. Dichloromethane (DCM, 10 mL) was then added, and the mixture was extracted for 30 min at 4 °C. The mixture was centrifuged for 5 min at 13,000 rcf, and the DCM layer was removed. DCM was evaporated under a gentle nitrogen stream, and crude residue was stored at -80 °C until LC-ESI/TOF analysis (see below).

Standard solutions (1 μM) of 3CPAA, 2FPAA, and 3BPAA were prepared. Standards were analyzed on an Agilent G1969A HPLC-ESI/TOF (see below) to generate calibration curve data. Injections were made corresponding to 0, 10, 50, and 100 pmoles of acid. To assess extraction efficiencies, the standard solutions were also carried through the DCM extraction procedure described above. Evaporated samples were analyzed by HPLC and compared to calibration curve data to estimate extraction efficiencies. Extraction efficiencies for all acids were ≥ 90%.

Mass spectrometry. LC-MS analysis was performed on an Agilent G1969A HPLC-ESI/TOF instrument. The solvents used were 5 mM ammonium formate, pH 3.8 (Solvent A) and 5 mM ammonium formate in 90% acetonitrile (Solvent B). The HPLC column was an Agilent Zorbax 1.8 μm C18 (2.1 mm x 50 mm). The HPLC gradient began at 2% Solvent B with a hold for 2 min, followed by a ramp to 90% Solvent B over 23 min. The mass spectrometer was used in negative ion mode, scanning over the m/z range 50–1700. The mass spectrometer source conditions included a gas temperature of 350 °C, drying gas flow rate at 10.0 L/min, nebulizer at 35 psig, electrospray voltage at 3500 V (negative ion mode), fragmentor at 105 V, skimmer at 60 V, and octopole RF at 250 V. Samples were prepared for analysis by dissolving extracted plant residue in a buffer consisting of 75% HPLC Solvent A and 25% Solvent B. Samples were then diluted 5-fold with HPLC Solvent A, and 10 μL of each sample was injected for LC-ESI/TOF analysis. The spectra were scanned for the presence of 3CPAA or 2FPAA ions (exact masses of 169.0056 or 153.0352 g/mol, respectively) to yield the EICs shown in Figure 7.

ASSOCIATED CONTENT

Supporting Information: Plant toxicity and additional growth assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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