SUPPORTING INFORMATION

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

Andrew G. Palmer,1,† Amanda C. Senechal,1,† Arijit Mukherjee,2 Jean-Michel Ané,2 and Helen E. Blackwell1,*

1Department of Chemistry, 1101 University Avenue, University of Wisconsin–Madison, Madison WI 53706; 2Department of Agronomy, 1575 Linden Drive, University of Wisconsin–Madison, Madison WI 53706

CONTENTS

• Additional text, biological data (Supplementary Figures 1–6), Supplementary Scheme 1, and assay methods in support of the main text.

† These authors contributed equally to this work.
* To whom correspondence should be addressed. blackwell@chem.wisc.edu
Supplementary Text.

Phytotoxicity assays. We performed a series of plant assays to examine any phytotoxicity associated with AHLs and establish an upper concentration limit for testing. OdDHL (8) was selected as a prototypical AHL. Three distinct variables were evaluated as indicators of plant stress in A. thaliana: (i) seed germination, (ii) total chlorophyll content, and (iii) ion (electrolyte) leakage (1-3). While ion leakage assays in A. thaliana are often performed on leaf discs, we evaluated ion leakage in roots, as this was the site of the most obvious phenotypic effect of applied AHLs. Aluminum chloride (AlCl₃), a common phytotoxicant, was included as a control in these phytotoxicity assays (at 100 µM) (4, 5). As seen in Supplementary Figures 2A–C, concentrations of OdDHL (8) < 200 µM had no impact on these common indicators of plant stress, while concentrations of OdDHL (8) ≥ 200 µM caused reduced germination, leaf chlorosis (loss of chlorophyll), and increased ion leakage from isolated roots (an indicator of poor cell membrane integrity). These effects increased with increasing OdDHL concentrations ≥ 200 µM. Based on these assay results, we established an upper concentration limit of 100 µM for testing AHLs in our root elongation assays.
Supplementary Figures and Scheme.

Supplementary Figure 1. Root elongation in *A. thaliana* is affected by compound exposure. Newly germinated *A. thaliana* seedlings were transferred to plates containing the indicated concentrations of compound (1–17), and primary root length was assessed after 14 d. Results for each compound treatment are expressed as the average of 20 measurements (n = 30), normalized to untreated samples (+/- standard error), and shown as different color bars in panels A–D. See main text for details of methods.
Supplementary Figure 2. Phytotoxicity assay data for OdDHL (8) in Arabidopsis thaliana. Seedlings were evaluated for the effects of the indicated concentration of OdDHL (8; white) or 100 µM AlCl₃ (grey) on (A) germination, (B) total chlorophyll content, or (C) ion leakage (see Supplementary Methods for details of assays). Experiments were performed in triplicate, and the results are expressed as the mean with error bars denoting standard deviation.
Supplementary Figure 3. Root elongation in *Medicago truncatula* is affected by compound exposure. Newly germinated *M. truncatula* seedlings were transferred to plates containing the indicated concentrations of AHL (1–8), and primary root length was assessed after 14 d. Results for each compound treatment are expressed as the average of 20 measurements (n = 20) and normalized to untreated samples (+/- standard error). These growth responses for *M. truncatula* were analogous to growth responses observed for *A. thaliana* when treated with AHLs 1–8 at 0.1 µM or 100 µM.
Supplementary Figure 4. Characterization data for 6XHis-AtFAAH. (A) Segment of the SDS-PAGE gel for nickel affinity resin-purified 6XHis-AtFAAH (mol. wt. 70 kD). Coomasie blue used for protein visualization. (B) Selected MALDI-TOF MS data for gel-excised AtFAAH after trypsin digest. Fragment masses for AtFAAH were predicted using Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm) and compared to masses obtained via MS analysis of trypsin-digested AtFAAH fragments. These data indicated that the purified protein was AtFAAH. See main text for details of methods.
**Supplementary Scheme 1.** Overview of putative enzymatic processing of AHLs into ACC (the direct ethylene precursor) after hydrolysis via AtFAAH in A. thaliana. AHL is hydrolyzed by AtFAAH, yielding L-homoserine. L-Homoserine is then phosphorylated via homoserine kinase (6). A series of enzymatic transformations carried out by cystathionine-\(\gamma\)-synthase (7, 8), cystathionine-\(\beta\)-lyase (9), and methionine synthase (10) ultimately yields L-methionine. Finally, consecutive transformations carried out by S-adenosylmethionine synthetase (11) and ACC synthase (12) give ACC, which is acted upon by ACC oxidase (13) to yield the phytohormone ethylene. The starred positions in the structures are conserved throughout the entire bioconversion of AHL to ACC. Thus, ACC derived directly from an AHL precursor should be identifiable through the use of a deuterated AHL analog (labeled with four deuterium atoms at the starred positions). We predict that three out of these four deuterium labels would be conserved; one C\(\beta\)-deuterium would be removed from phosphorylated L-homoserine in processing by cystathionine-\(\gamma\)-synthase (8, 14). Consequently, deuterated AHL should yield “heavy” ACC with a mass of M+3 (6, 8, 10-16).
**Supplementary Figure 5.** The *M. truncatula* Δskl mutant is resistant to the effects of AHLs on growth. Newly germinated *M. truncatula* seedlings (wild-type or Δskl) were transferred to plates supplemented with either OdDHL (8) or L-homoserine (16) (at 0.1 or 50 μM), and primary root length was assessed after 14 d. Results for each compound treatment are expressed as the average of 20 measurements (n = 20) and normalized to untreated samples (+/- standard error). These results indicate a reduced response to both compounds at 0.1 μM and an increased response to both compounds at 50 μM in the ethylene-insensitive mutant Δskl as compared to wild-type *M. truncatula*, suggesting that ethylene is important for AHL-induced growth effects in *M. truncatula*. **“*” = p-value < 0.05 relative to wild-type (wt) samples treated with 0.1 μM of the corresponding compound (8 or 16).**
Supplementary Figure 6. Amidolysis data for the indicated compound (at 100 µM) by purified human FAAH as monitored by the reaction of L-homoserine (16) with fluorescamine (see main text for methods). Compounds 4, 8, 11, 12, and 14 all have a 12-carbon acyl tail and are indicated in grey for comparison (note, 12 is the D-enantiomer of 8 (OdDHL)). Pos (Positive control) = linoleoyl ethanolamide (100 µM); Neg (Negative control) = DMSO.
Supplementary Methods.

Seed germination assay protocol. Seeds of *A. thaliana* (wt) were surface sterilized (5 min, 70% EtOH) and transferred in 50 seed aliquots to germination plates (0.1x MS and 3 g/L gelzan – a common gelling agent) supplemented with either OdDHL (8) or AlCl$_3$. Plates were sealed with parafilm and stored in the dark at 4 °C for 72 h. Following this imbibing time, the seeds were transferred to a room temperature dark storage area (a drawer) and scored for germination after 72 h.

Total chlorophyll and ion leakage assay protocols. In order to acquire sufficient plant material for total chlorophyll and root ion leakage assays, germinated seedlings of *A. thaliana* (wt) were grown for 10 d on AHL-free plates (0.5x MS, 30 g/L sucrose, and 4 g/L gelzan), then transferred to growth plates containing OdDHL (8) or AlCl$_3$ and grown for an additional 7 d (see main text for standard growth conditions). Thereafter, the aerial portions and roots of plants were separated and analyzed for total chlorophyll and ion leakage, respectively, as described below.

The total chlorophyll content assay was based on a previously described protocol used to monitor phytotoxicity (17). The aerial portions of *A. thaliana* seedlings ($n = 10$) were collected and their combined mass determined using a Mettler Toledo XS105 analytical balance. Samples were submerged in 4:1 acetone/H$_2$O (10 mL) and gently shaken at 4 °C in the dark for 4 h to extract chlorophyll. A 1 mL aliquot was removed from the solution, and chlorophyll a & b absorbance was measured at 645 nm and 663 nm using a Jenway 7305 spectrophotometer. Total concentration of chlorophyll a & b in the plant sample was calculated using the following equation:

$$\text{Total chlorophyll (mg/g of sample) = } [20.2(\text{Abs}_{645})][8.02(\text{Abs}_{663})]V/1000W$$

$V =$ Final volume of 80% acetone, $W =$ weight of sample
Our root ion leakage assay protocol was based on previously reported methods (1, 2, 18, 19). Freshly cut *A. thaliana* seedling roots (*n* = 10) were immediately transferred to 7.5 mL of sterile dH₂O, capped, and gently shaken at room temperature for 12 h. A 5 mL sample was removed and a conductivity measurement was collected using a Cole-Parmer microvolume conductivity meter to monitor initial ion leakage. The sample was boiled for 10 min, allowed to cool to room temperature, and a second conductivity measurement was collected (total ion). The results were expressed as percent ion leakage (% ion leakage = initial ion leakage/total ion).

**A. thaliana transpiration assay protocol.** Our assay protocol was based in part on that of Kuromori *et al.* (20). Germinated seedlings of *A. thaliana* (wt or ∆AtFAAH) were transferred to strips of sterile filter paper in test tubes. Samples were grown in 0.5x MS media with a 16:8 h light/dark cycle for 14 d to ensure sufficient leaf stock. The media was then exchanged for fresh MS media containing the compound of interest. After 48 h of compound exposure, 5 leaves were excised, and the subsequent loss of mass due to transpiration was evaluated at 30 min intervals over a 5 h period by weighing the leaves on a Mettler Toledo XS105 analytical balance.
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