Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals

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Many bacteria use *N*-acyl homoserine lactone (AHL) signals to coordinate the behavior of individual cells in a local population. The successful infection of eukaryotic hosts by bacteria seems to depend particularly on such AHL-mediated "quorum-sensing" regulation. We have used proteome analysis to show that a eukaryotic host, the model legume *Medicago truncatula*, is able to detect nanomolar to micromolar concentrations of bacterial AHLs from both symbiotic (*Sinorhizobium meliloti*) and pathogenic (*Pseudomonas aeruginosa*) bacteria, and that it responds in a global manner by significant changes in the accumulation of over 150 proteins, 99 of which have been identified by peptide mass fingerprinting. The accumulation of specific proteins and isoforms depended on AHL structure, concentration, and time of exposure. AHLs were also found to induce tissue-specific activation of β-glucuronidase (*GUS*) reporter fusions to an auxin-responsive and three chalcone synthase promoters, consistent with AHL-induced changes in the accumulation of auxin-responsive and flavonoid synthesis proteins. In addition, exposure to AHLs was found to induce changes in the secretion of compounds by the plants that mimic quorum-sensing signals and thus have the potential to disrupt quorum sensing in associated bacteria. Our results indicate that eukaryotes have an extensive range of functional responses to AHLs that may play important roles in the beneficial or pathogenic outcomes of eukaryote–prokaryote interactions.

Plant and animal hosts need to detect the presence of bacteria quickly and reliably to make appropriate responses to pathogens or symbionts. Many bacteria are known to depend on the exchange of *N*-acyl homoserine lacton (AHL) signals to monitor changes in the number and proximity of siblings and coordinately activate new gene expression during host infection (1–4). Thus it is important to know whether eukaryotes, in turn, have evolved the means to detect bacterial AHLs and respond appropriately. Earlier studies have indicated that animal cells do respond to added AHLs in several specific ways. A well known AHL from the environmental-pathogenic bacterium *Pseudomonas aeruginosa*, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide (=3-oxo-C_{12}-HL) (5), was found to have immunomodulatory effects on macrophage and T helper cell responses in mammalian cells (6), to inhibit expression of cystic fibrosis gland cell P2Y receptors (7), to inhibit porcine smooth muscle contraction (8), and to affect IL-8 production in human cells through transcriptional regulation by NF-κB and activator protein-2 (9). More recently, it was reported that s.c. injection of 3-oxo-C_{12}-HL stimulated a number of inflammatory and immunogenic responses in mice (10), suggesting that AHLs may act as virulence factors.

In this study, proteomic analysis was used as a general and global approach to detect the responses of a eukaryote to bacterial quorum-sensing signals. We hoped to learn whether plants were also able to detect and respond to bacterial AHL signals, whether the responses were narrow or extensive, and whether different bacterial signals would elicit different responses in the host. The model legume *Medicago truncatula*, a close relative of alfalfa, was chosen as the eukaryotic host. It forms a well studied symbiotic relationship with the AHL-producing nitrogen-fixing bacterial symbiont *Sinorhizobium meliloti* (11) and is exposed to many saprophytic and pathogenic soil bacteria, including *Pseudomonas* spp. To test the responses of *M. truncatula* to AHLs, seedlings were treated with either 3-oxo-C_{12}-HL, produced by *P. aeruginosa* or the recently identified AHL, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)hexadecenamide (=3-oxo-$C_{16:1}$HL) produced by *S. meliloti* (12).

Materials and Methods

Plant Growth and Treatments. *M. truncatula* genotype A17 was grown aseptically from surface-sterilized seeds on agar plates under controlled growth conditions (13). Roots of 3-day-old seedlings were treated with either 3-oxo-C^{12}-HL (Quorum Science, Corville, IA) or 3-oxo-$C^{16:1}$-HL (extracted from early stationary phase culture filtrates of *S. meliloti* 1021 and purified three times by reverse-phase HPLC). Agar blocks ($5 \times 5 \times 2$ mm) containing the AHLs were placed on top of the root in the zone of root hair elongation and removed after 24 or 48 h. Alternatively, the seedlings were sprayed with AHL solutions by using an atomizer. AHLs in ethyl acetate were diluted 10^3- to 10^5-fold to the desired concentration with sterile water. Control roots were treated with agar blocks or spray containing equivalent dilutions of ethyl acetate in water. The concentration of 3-oxo-$C_{16:1}$-HL is not known with certainty. On the basis of bioassay comparisons to determine the threshold of detection for 3-oxo-$C_{16:1}$-HL and 3-oxo-C_{12}-HL by using the pSB1075 AHL reporter (14), the highest concentration of 3-oxo-$C_{16:1}$-HL used here is estimated to be ≈1–10 nM (data not shown). Approximately 200 root segments, covering the treated root section only, were harvested directly into liquid nitrogen for each gel. All experiments were repeated three times with three independent gels for each batch of roots.

Proteome Analysis. Proteins were extracted and then separated by 2D PAGE, as described (13). The gels showed ≈2,000 spots in the pH range of 4–7 on Coomassie-stained gels. Gels were scanned at 600 dpi on a UMAX 2400 Astra scanner (Scanner-Place, Canberra, Australia), and relative protein abundance was quantified with MELANIE 3.05 software (Swiss Institute of Bioinformatics, Geneva) on three gels for each treatment. ANOVA (using a P value of $P < 0.05$) was performed with GENSTAT software (Version 5.0, Lawes Agricultural Trust, Rothamstead, U.K.) to test for statistical significance of differences in protein abundance between the different treatments. Proteins were identified from Coomassie-stained gels by peptide mass fingerprinting after tryptic digestion and matrix-assisted laser desorption ionization–time of flight MS performed on a Micromass TofSpec 2E Time of Flight Mass Spectrometer (Waters) at the Australian Proteome Analysis Facility (Macquarie University, Sydney). Peptide mass fingerprints were identified by comparison against the *M. truncatula* tentative cluster (TC) database (www.tigr.org/tdb/tgi/mtgi/) by using masslynx software (Micromass, Waters), as described (13). The TC annotations provided by The Institute for Genomic Research (TIGR) and the respective

Abbreviations: AHL, *N*-acyl-homoserine lactone; GUS, β-glucuronidase.

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Tentative Ortholog Groups were used for the prediction of protein functions shown in Table 1 and Table 2, which is published as supporting information on the PNAS web site, www.pnas.org.

Gene Expression Assays in White Clover. Transgenic white clover plants were generated in previous studies (15, 16) and contained fusions of the β-glucuronidase (*GUS*) reporter gene with either the soybean auxin-responsive promoter, *GH3*, or with one of three clover chalcone synthase promoters (*CHS1*, *CHS2*, or *CHS3*). Roots were generated from leaves of mature transgenic plants by using the rooted leaf assay (17) to produce clonal root material with homogenous expression patterns. Each plant contained a single copy of the transgene and expressed consistently between individuals (15, 16). At least two transformed lines were analyzed for each construct. Roots were spotted in the zone of emerging root hairs with a 50-nl droplet of 50 μM 3-oxo-C_{12}-HSL and incubated for 24 h in a growth cabinet. Between 15 and 30 roots were used for histochemical assays, and between 40 and 60 roots were pooled for fluorometric assays. All assays were repeated at least twice. Fluorometric assays used methyl-umbelliferone glucoronide as a substrate and Fluoroscan II (Labsystems, Chicago) for quantification. Histochemical GUS assays were done as described (15).

Detection of Quorum-Sensing Signal-Mimic Activities in Exudates. *M. truncatula* seeds were surface sterilized, imbibed overnight at 4°C, and resterilized briefly. Seedlings were grown aseptically in the light in a shallow pool of water at 26°C for 6 d. Sets of 400–600 seedlings were either untreated or exposed to 2 nM C_6-HL (Quorum) or 3-oxo-C_{12}-HL. After 48 h, the exudate solution from each tray was tested for microbial contamination, discarded if contaminated, then extracted with an equal volume of ethyl acetate. Ethyl acetate extraction reduced the concentration of added C_6-HL and 3-oxo-C_{12}-HL to below detectable levels by bioassay. The aqueous phase was freeze-dried and extracted with methanol. Two-milliliter samples containing compounds from ≈300–500 seedlings were subjected to reverse-phase HPLC on a semipreparatory C_{18} column (Whatman Partisil 10, ODS-3) equilibrated with 10:90 acetonitrile/water. Samples were eluted at 1 ml/min with a linear acetonitrile/water gradient. The *Escherichia coli* HB101 pSB1075 (*lasRI′ luxBCDAE*) AHL reporter (cognate signal = 3-oxo-C_{12}-HL) and the *Vibrio harveyi* BB170 reporter (cognate signal = AI-2) were used to detect quorum-sensing active compounds in the fractions essentially as described (14, 18). A volume of each HPLC fraction equivalent to 15–40 seedlings was transferred to a microtiter plate well, dried, mixed with 80 μl of reporter suspension, and luminescence measured after ≈3 h with a Victor2 microtiter plate reader [Wallac (Gaithersburg, MD), Perkin–Elmer].

Results and Discussion

Proteome Analysis of AHL-Treated Roots. Roots of axenically grown *M. truncatula* were exposed to low concentrations (10 nM–2 μM) of 3-oxo-C_{12}-HL or to comparable concentrations (≈1–10 nM) of 3-oxo-$C_{16:1}$-HL for either 24 or 48 h. Root segments were then excised and the extracted proteins separated by 2D PAGE and identified by peptide mass fingerprinting matching against the *M. truncatula* tentative cluster database, as we have done previously to establish the *M. truncatula* root proteome reference map (13).

Exposure to these two AHLs reproducibly and significantly changed the accumulation level of 154 proteins. Table 1 provides a list of 99 of these proteins with homologs in the database. In addition, exposure to the AHLs caused significant changes in the accumulation of 47 other proteins that had no good homology to known proteins as well as another eight proteins that responded only to high (50 μM) concentrations of 3-oxo-C_{12}-HL (Table 2). Both of the AHLs tested had strong effects on the levels of individual proteins. AHL-induced changes in level were greater than 4-fold for ≈75% of the proteins listed in Table 1. One would expect such substantial changes in protein accumulation to result in correspondingly large changes in the associated functions.

Responses to AHLs from two different bacterial species were examined to determine whether eukaryotic hosts such as *M. truncatula* might be able to recognize frequently encountered bacterial symbionts or pathogens based on the bacterium's use of particular AHLs. The two AHLs tested here were found to have qualitatively, quantitatively, and temporally similar effects on the accumulation of about two-thirds of the 99 proteins in Table 1. This may indicate that the plant has one fairly basic set of responses to AHLs regardless of exact AHL structure. However, the remaining one-third of the proteins showed responses to the two AHLs that were distinct in terms of either the magnitude or direction of change in accumulation or the time of maximum response. These differences suggest that the plant is able to distinguish between AHLs of fairly similar structure and then respond to each in different ways.

The plant showed significant changes in response to AHLs between 24 and 48 h. At 24 h, the levels of most proteins in Table 1 were significantly reduced in response to AHL treatment, followed by increased accumulation of many of these proteins by 48 h. These general temporal changes in protein level may be related to the considerable number of AHL-responsive proteins with putative roles in proteolysis or protein processing (Table 1). There may also be potent temporal changes in the auxin balance in roots exposed to AHLs, as indicated by the appearance of an auxin-induced protein (TC51487) at 48 h and by the large increase in accumulation of an auxin-deactivating enzyme, indole-3-acetate-β-glucosyltransferase. Auxin (indole-3-acetic acid) is one of the most important plant hormones, with roles in many aspects of plant growth, development, and symbiosis (19, 20).

AHL concentration was an important factor in certain plant responses, but significant responses were seen at all concentrations tested, from about 1 nM to 50 μM, covering the range used for quorum sensing by bacteria *in vitro* (21). Exposure to a high (50 μM) concentration of 3-oxo-C_{12}-HL led to significantly increased accumulation of 60 of the 154 proteins, even though 53 of these were significantly diminished on exposure to lower concentrations (Table 2). This suggests that the host plant may have a different pattern of response to low AHL levels than it does to higher AHL levels that might reflect an acute or extensive exposure to bacteria. The AHLs elicited similar responses when provided in agar blocks or a spray, indicating that effective detection and response by the plant did not require the sustained exposure to an AHL that is provided by a block.

The responses of *M. truncatula* to AHLs were quite extensive in terms of the percentage of the total resolved proteins that were affected (≈6%) and in terms of the diversity of functions represented. On the basis of homology to known proteins, ≈23% had functions plausibly related to plant defense or stress responses; 14% to protein degradation or processing; 5% to flavonoid synthesis; 5% to plant hormone responses or synthesis; 10% to regulatory functions; 6% to cytoskeletal elements; and 37% to energetics and various primary metabolic activities (Table 1). Because only the most abundant proteins were detected, it is likely that AHLs also affect many other functions and regulatory elements. Future genomic transcriptional studies should provide valuable complementary information concerning the range and pattern of eukaryotic responses to AHLs.

Induction of Reporter Genes by AHLs. To test whether AHLs induced changes in gene expression related to some of the protein level changes seen in Table 1, we analyzed both auxin-induced gene expression, as suggested by changes in proteins like TC51487, and flavonoid-related gene expression, as suggested by differential accumulation of isoflavone reductases, vestitone

Table 1. Proteins differentially accumulated in AHL-treated roots as percent of controls

Putative identity	M.t. TC‡	TOG§	pI/M_r¶	C12 ** 2 uM block 24h	C12 100 nM spray 24h	C12 10 nM‡‡ spray 24h	C16 10 nM§§ block 24h	C16 1 nM§§ spray 24h	C12 2 uM block 48h	C16 10nM§§ spray 48h
Defense and stress response *										
L-ascorbate peroxidase, cytosolic	TC43117	129246	5.9 / 26	10 ††	10	10	70	20	80	130
L-ascorbate peroxidase, cytosolic	TC43117	129246	5.0 / 25	60	10	3	40	10	80	170
L-ascorbate peroxidase, cytosolic	TC43117	129246	6.1 / 32	20	20	20	70	30	70	100
Glucan endo-1,3-beta-glucosidase	TC45439	n/a	6.7 / 33	310	90	230	100	90	80	70
Glutathione dependent dehydroascorbate reductase	TC51525	129252	5.9 / 29	20	4	60	50	20	90	100
Hypersensitive-induced response protein (HIR1)	TC43552	129912	5.2 / 30	10	40	100	50	20	20	0
similar to Late embryogenesis abundant (LEA) protein	TC26214	156708	4.7 / 37	60	20	40	100	60	110	150
Superoxide dismutase	TC43738	126480	6.5 / 24	10	10	70	50	5	70	130
Peroxidase	TC52384	158767	6.6 / 36	20	20	30	90	30	90	150
Thioredoxin peroxidase	TC43680	128471	5.9 / 17	50	20	40	80	50	160	110
Thioredoxin peroxidase	TC43680	128471	5.6 / 18	150	160	110	250	150	160	160
Phospholipid hydroperoxide glutathione peroxidase	TC51659	128476	6.3 / 18	60	30	70	90	40	80	70
PR10-1 protein	TC51061	n/a	4.3 / 15	40	50	60	120	60	60	80
PR10-1 protein	TC51061	n/a	5.1 / 16	120	110	140	150	190	70	100
PR10-1 protein	TC51061	n/a	4.4 / 17	50	60	70	110	50	140	50
PR10-1 protein	TC51061	n/a	4.5 / 17	50	30	60	120	60	60	40
Putative Hs1pro-1 homolog	TC51338	140039	6.2 / 29	80	20	20	90	60	150	270
similar to Ripening related protein†	TC51327	n/a	5.5 / 18	80	60	80	150	100	80	60
similar to Ripening related protein†	TC51851	n/a	6.2 / 17	40	7	70	160	4	1110	5110
similar to Ripening related protein†	TC51326	n/a	5.9 / 18	0	0	250	140	0	40	40
similar to Ripening related protein†	TC51330	n/a	5.9 / 17	30	20	30	170	50	40	130
similar to Ripening related protein†	TC21961	n/a	5.5 / 18	new	new	new	new	new	210	200
similar to Ripening related protein†	TC21963	n/a	5.9 / 18	20	6	20	50	10	90	80
Flavonoid metabolism										
Chalcone isomerase	TC45048	131607	6.1 / 25	30	30	40	80	30	140	200
Isoflavone reductase	TC43281	n/a	5.8 / 33	30	20	30	60	30	120	140
Isoflavone reductase	TC43281	n/a	5.6 / 33	60	50	110	30	80	150	700
Isoflavone reductase	TC43281	n/a	5.5 / 37	190	90	290	780	300	880	110
Isoflavone reductase-like NAD(P)H-dependent oxidoreductase	TC43869	130227	6.6 / 33	50	10	110	110	40	90	100
Vestitone reductase	TC51930	174422	4.4 / 21	20	10	20	30	20	50	50
Hormone response and metabolism										
ABA-responsive protein	TC43134	n/a	4.8 / 16	60	50	70	110	70	70	80
2,4-D inducible glutathione S-transferase	TC51307	133964	6.4 / 26	30	10	100	70	20	90	100
Putative auxin-induced protein	TC51487	130978	6.1 / 37	60	0	50	100	30	new	new
Auxin- or ripening induced protein	TC45813	157407	5.5 / 34	80	70	40	70	100	110	50
Auxin-induced protein (Quinone oxidoreductase-like protein)	TC44469	133822	6.1 / 36	40	30	70	140	50	150	130
Indole-3-acetate beta-glucosyltransferase	TC45997	n/a	5.6 / 44	20	60	30	20	20	1030	1810
Regulatory proteins										
Adenylate kinase-a	TC51802	126968	6.8 / 29	240	190	380	160	10	new	0
probable DNA-binding protein	TC43627	127452	6.7 / 18	200	0	240	0	200	0	0
Early nodulin ENOD18 (ATP binding protein)	TC43493	133037	6.3 / 17	40	10	60	70	40	110	110
Translation Elongation factor 1-beta	TC43535	126576	4.2 / 32	70	100	50	100	50	120	70
Nucleoside diphosphate kinase 1	TC43358	126390	6.6 / 15	40	20	60	90	50	50	80
Poly (A) binding protein	TC43026	126489	5.3 / 18	140	90	90	130	260	80	50
putative transcription factor BTF3	TC51530	127518	6.8 / 22	240	70	120	330	0	0	new
RNA binding protein 45	TC51432	169345	6.4 / 27	0	30	60	100	10	100	360
Protein degradation										
Cysteine proteinase precursor	TC43256	127013	4.3 / 38	10	100	10	90	20	0	0
Cysteine proteinase inhibitor (cystatin)	TC44534	130947	6.4 / 26	50	20	50	100	50	90	170
Cysteine proteinase inhibitor (cystatin)	TC44546	132105	6.3 / 10	150	120	650	420	130	60	140
Protease inhibitor	TC15512	n/a	4.7 / 21	690	490	870	350	980	130	270
Proteasome subunit alpha type 5	TC52108	126530	4.4 / 25	60	50	70	90	80	90	50
Proteasome subunit alpha type 1-2	TC43700	126692	5.2 / 34	30	20	110	260	100	170	10
Proteasome subunit alpha type 6	TC43840	126527	5.9 / 26	30	20	30	60	30	110	110
Thiolprotease or cysteine proteinase	TC51274	127480	4.4 / 31	30	50	100	380	0	100	240
Protein synthesis and processing										
20 kDa chaperonin chloroplast precursor (Protein Cpn21)	TC44234	159458	5.7 / 24	160	60	100	160	200	90	110
Mitochondrial processing peptidase beta subunit	TC51456	126663	5.2 / 50	20	60	40	60	40	150	70

reductase, and chalcone isomerase. The effects of AHLs on gene expression were tested by applying a 50-nl droplet of AHL solution onto the root of a transgenic white clover (*Trifolium repens*) plant with a relevant GUS reporter fusion. As shown in Fig. 1, 24-h exposure to 3-oxo-C_{12}-HL substantially increased expression of the auxin-inducible *GH3* promoter as well as the

Table 1. (continued)

Putative identity	M.t. TC[‡]	TOG[§]	pI/M_r[¶]	C12 ** 2 uM block 24h	C12 100 nM spray 24h	C12 10 nM[‡‡] spray 24h	C16 10 nM[§§] block 24h	C16 1 nM[§§] spray 24h	C12 2 uM block 48h	C16 10nM[§§] spray 48h
Mitochondrial processing peptidase alpha-II chain precursor	TC52124	132899	6.3 / 60	60	30	60	120	4	230	510
Nascent polypeptide associated complex α chain	TC51504	127007	4.1 / 34	160	510	170	530	480	240	50
Nascent polypeptide associated complex α chain	TC51504	127007	4.2 / 29	300	150	120	80	370	160	80
Protein disulfide isomerase precursor	TC43394	126701	4.8 / 58	40	30	30	120	50	80	110
Protein disulfide isomerase precursor	TC43394	126701	4.7 / 58	80	20	70	130	40	50	80
Cytoskeleton										
Actin-depolymerizing factor 2	TC43253	180082	6.6 / 16	60	40	140	190	20	870	960
Tubulin alpha-1 chain.	TC51281	126302	5.0 / 50	0	100	140	330	310	660	250
Tubulin alpha-1 chain.	TC51283	n/a	5.0 / 50	30	70	80	150	30	470	120
Tubulin alpha-3/alpha-5 chain	TC51280	129723	5.1 / 50	30	40	70	90	50	270	70
Tubulin beta chain	TC43237	144406	4.4 / 52	90	140	170	140	150	200	0
Tubulin beta chain	TC43242	126299	4.3 / 52	70	110	110	130	100	110	0
Various metabolic functions										
Alpha-fucosidase	TC54892	n/a	4.7 / 22	150	50	90	100	100	150	0
probable Aminoacylase	TC52126	127533	6.4 / 50	0	20	40	100	20	new	new
ATP synthase beta chain mitochondrial precursor	TC43266	126419	5.4 / 54	40	30	40	60	60	140	30
ATP synthase beta chain mitochondrial precursor	TC43266	126419	5.3 / 50	50	40	60	70	60	120	70
Caffeoyl-CoA O-methyltransferase	TC43382	129263	5.4 / 28	30	20	40	60	50	80	70
Carbonic anhydrase	TC51755	143705	5.7 / 28	80	50	30	220	50	160	230
Cytochrome c oxidase subunit 6	TC44787	130851	4.2 / 37	50	40	60	80	70	40	40
Enolase	TC51195	126309	4.5 / 28	80	30	20	70	10	200	70
Enolase	TC51195	126309	5.6 / 50	30	20	30	80	10	140	100
Enolase	TC51195	126309	5.7 / 50	30	30	30	60	20	130	60
Enolase	TC51195	126309	5.5 / 46	30	10	40	70	20	150	110
Fructokinase	TC43388	129738	5.2 / 35	50	50	70	110	50	140	90
Fructose-1,6-bisphosphate aldolase, cytosolic	TC51103	126371	6.8 / 38	50	20	100	140	20	100	90
Fructose-1,6-bisphosphate aldolase, cytosolic	TC51103	126371	6.6 / 39	30	20	60	90	30	100	110
Fructose-1,6-bisphosphate aldolase, cytosolic	TC51103	126371	6.4 / 38	30	20	30	180	20	260	280
Glyceraldehyde-3-phosphate dehydrogenase	TC43151	126306	6.8 / 37	30	10	100	70	0	100	70
Glyceraldehyde-3-phosphate dehydrogenase	TC43151	126306	6.8 / 37	50	10	40	70	0	240	130
Glyceraldehyde-3-phosphate dehydrogenase	TC43151	126306	5.9 / 28	0	50	70	150	0	110	210
GPDH breakdown product	TC43151	126306	4.4 / 10	450	170	460	280	530	80	80
Glyoxalase II cytoplasmic isozyme	TC45468	127542	5.5 / 30	15	30	40	170	20	70	80
Malate dehydrogenase, cytosolic	TC43296	139607	6.2 / 35	60	70	60	120	70	150	220
Methionine synthase	TC43181	127389	6.5 / 80	10	0	90	10	0	new	new
Myo-inositol 1-phosphate synthase 1	TC51340	126526	5.7 / 55	20	30	50	40	20	250	150
NADP-dependent malic enzyme	TC43360	126594	6.4 / 65	10	20	40	80	3	100	280
2-oxoglutarate dehydrogenase E2 subunit	TC43810	126962	6.4 / 43	3	4	20	10	0	100	120
Phosphoglyceromutase	TC51208	129651	5.6 / 60	20	20	20	50	10	150	220
Phosphoglyceromutase	TC51208	129651	5.7 / 60	30	20	30	50	10	150	240
Oxygen-evolving enhancer protein 1 chloroplast precursor	TC43209	128629	5.0 / 29	40	140	90	100	120	60	0
RUBISCO small subunit	TC43022	127791	6.7 / 14	3700	880	630	5050	700	20	60
S-adenosyl-L-methionine synthetase 1	TC51026	126388	5.4 / 42	20	20	20	80	20	150	60
S-adenosyl-methionine synthase 2	TC51020	126472	6.2 / 41	10	20	10	80	20	120	60
S-adenosylmethionine synthase 3	TC46454	n/a	6.0 / 42	5	10	6	30	10	140	60
Triosphosphate isomerase	TC43332	126436	6.0 / 26	40	20	40	90	40	130	90
UDP-glucose pyrophosphorylase	TC51402	126613	5.2 / 45	20	0	120	140	0	50	0
UDP-glucose pyrophosphorylase	TC51402	126613	5.2 / 50	90	70	70	100	80	170	60

*Proteins are grouped by putative function, recognizing that some functions may belong in more than one group.

[†]This protein shows homology to a ripening induced protein of unknown function but is also inducible in soybean by the soybean-cyst nematode and so may be defense-related in roots.

[‡]The *M. truncatula* (M.t.) tentative clusters (TCs) against which the proteins were identified. These can be accessed at www.tigr.org/tdb/tgi/mtgi/.

[§]TOG indicates the Tentative Ortholog Group into which the *M. truncatula* TCs have been classified by The Institute for Genomic Research (TIGR). Access is through www.tigr.org/tdb/tgi/mtgi/. n/a indicates that no TOG has been assigned to this TC.

[¶]The observed M_r and pI for each *M. truncatula* protein were determined from its position on the gels relative to known marker proteins.

**−Cl2 = 3-oxo-C_{12}-HL; C16 = 3-oxo-$C_{16:1}$-HL.

[††]Relative accumulations are given in percent of the control level and are averages of results from three individual control gels and three individual treatment gels. Accumulation levels are also indicated by color code: yellow, a new protein not detected in controls; red, significantly increased; tan, nonsignificantly increased; pale green, nonsignificantly reduced; dark green, significantly reduced; blue, reduced below detection. *P* values for statistical significance of difference from control levels ranged from <0.001 to <0.005 but were <0.002 for $>90\%$ of the proteins listed.

[‡‡]Only two gels were examined for proteins from roots exposed to the lowest concentration (10 nM) of 3-oxo-C_{12}-HL.

[§§]Estimated concentration; see *Materials and Methods*.

three chalcone synthase (*CHS*) promoters. Induction of these promoters by 3-oxo-C_{12}-HL was not limited to, or highest in, the epidermal cell layer (Fig. 1), as might be expected if responses to AHLs were an artifact of membrane disruption. Induction of gene expression by 3-oxo-C_{12}-HL was seen in all cell types in the *GH3*:*GUS* plants and was not restricted to cells at the local site

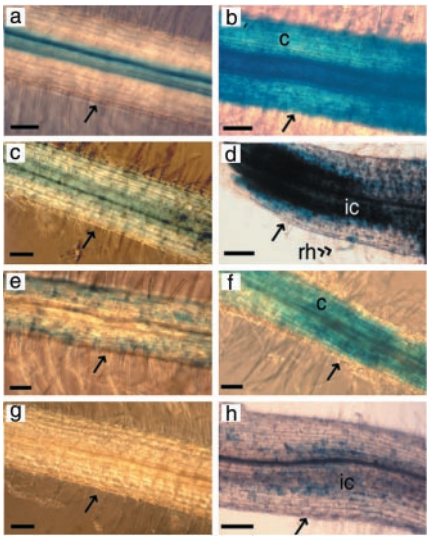

Fig. 1. Transgene induction in white clover roots by AHLs. Histochemical GUS staining of control roots (*a*, *c*, *e*, and *g*) or roots treated for 24 h with 50 nl of 50 μM 3-oxo-C_{12}-HL (*b*, *d*, *e*, and *h*) show induction of *GUS* fusions of the auxin responsive promoter *GH3* (*a* and *b*) and the three chalcone synthase promoters, *CHS1* (*c* and *d*), *CHS2* (*e* and *f*), and *CHS3* (*g* and *h*). The arrow indicates the site of droplet application. Staining patterns were consistent in repeat experiments with 12–33 roots per treatment. Quantitative fluorometric assays confirmed histochemical staining results and showed induction of transgene expression from 150% to 240% of control levels (data not shown). c, cortex; ic, inner cortex; rh, root hair. (Bar = 0.5 mm.)

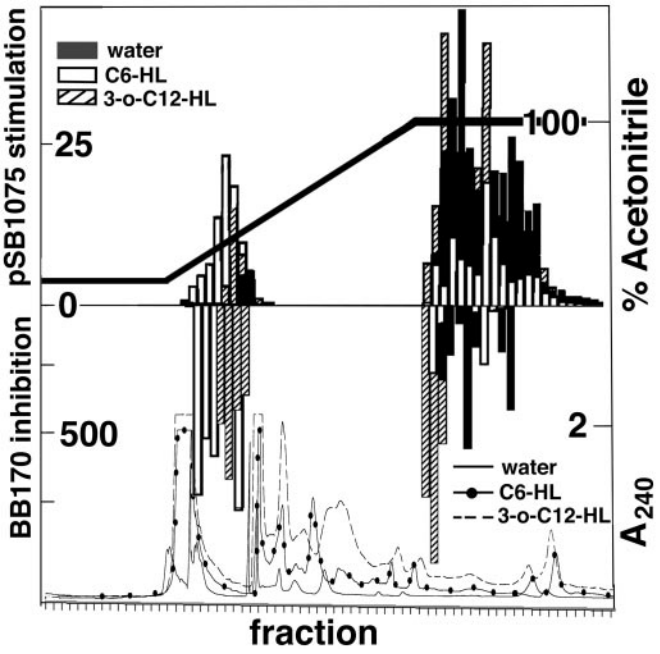

Fig. 2. AHL effects on host secretion of quorum-sensing signal mimics. Methanol extracts of exudates from seedlings exposed to 2 nM C_6-HL, 2 nM 3-oxo-C_{12}-HL, or water were fractionated by reverse-phase HPLC as described in *Materials and Methods*. Aliquots of individual fractions were bioassayed with the *E. coli* pSB1075 (*lasRI′*::*luxCDABE*) AHL reporter to detect plant compounds that stimulate luminescence by mimicking AHL signals (*Upper*) and with the *V. harveyi* BB170 reporter to detect plant compounds that inhibit the luminescence induced by added AI-2 (*Lower*), as described in *Materials and Methods*. Luminescence responses to compounds present in each fraction relative to reporter-only control values are indicated for the different treatments by bars (solid, water control; open, C_6-HL; hatched, 3-oxo-C_{12}-HL). The heavy line (*Upper*) indicates the acetonitrile concentration present in the collected fractions. The UV absorbance (0–2.0) of compounds present in methanol extracts from seedling exudates that were not preextracted with ethyl acetate was monitored during HPLC fractionation at 240 nm (*Lower*). Results shown are from one experiment representative of three independent trials.

of AHL application. In contrast, the *CHS1, CHS2,* and *CHS3* promoters were activated only near the site of AHL application and primarily in cortical cells, especially inner cortical cells in the case of *CHS3* (Fig. 1). Thus, exogenous AHLs elicited responses that were quite tissue- and gene-specific. Exposure of the white clover transgenics to 1–10 nM 3-oxo-$C_{16:1}$-HL gave very similar responses (data not shown). Although proteins identified by homology may not share the same function or regulation in other organisms, it is noteworthy that proteins identified as AHL-responsive in *M. truncatula* could be used as guides to predict AHL-responsive genes in another species (white clover). Correspondence of this sort should facilitate the future characterization of plant and animal responses to AHLs by tests in species were appropriate reporters are available.

Plant Secretion of AHL Mimics. We also looked for AHL-induced changes in the secretion of host metabolites that might specifically affect interactions of the host with bacteria. *M. truncatula* is among several plant species that were found to secrete AHL "signal-mimic" substances of unknown structure that specifically stimulate or inhibit AHL-regulated responses in bacteria (22) and thus have the potential to manipulate bacterial behaviors regulated by quorum sensing (22, 23). Exudates were collected from *M. truncatula* seedlings after 48-h exposure to 2 nM 3-oxo-C_{12}-HL or C_6-HL. C_6-HL is the principal AHL produced by certain *Chromobacterium*, *Yersinia*, and *Rhizobium* strains (2) and was selected for testing to learn whether an AHL with a short undecorated side chain would elicit responses comparable to an AHL with a long decorated side chain.

The UV absorbance profiles obtained after reverse-phase fractionation of the methanol soluble compounds in exudates from AHL- and water-treated seedlings showed differences in the sizes and locations of several peaks (Fig. 2 *Lower*). This indicates that low concentrations of both 3-oxo-C_{12}-HL and C_6-HL affected the kinds and amounts of UV absorbing metabolites secreted by the plant.

Two bacterial reporter strains designed to detect exogenous quorum-sensing signals (*E. coli* pSB1075 and *V. harveyi* BB170) were used to compare the HPLC fractions from untreated and AHL-treated plant exudates. Substantial activities corresponding to plant-derived compounds that mimic bacterial quorum-sensing signals were detected (Fig. 2). The profile of stimulatory activities seen with the pSB1075 reporter was almost a mirror image of the inhibitory activities seen with the BB170 reporter, suggesting that both reporters may be responding in opposite ways to the same set of plant compounds. The plant compounds stimulated the pSB1075 (LasR) AHL reporter about 20- to 50-fold, comparable to the stimulation that would be elicited by addition of ≈5 nM levels of the cognate AHL, 3-oxo-C_{12}-HL. The plant compounds inhibited the responses of the BB170 reporter to low levels of added AI-2 by roughly 20- to 900-fold. AI-2 is a furanosyl borate ester (24), not an AHL. It is used as a quorum-sensing signal by *V. harveyi* and perhaps a variety of other bacteria (2). The results in Fig. 2 provide evidence that plants secrete substances that affect AI-2-dependent quorum sensing in bacteria. The secretion of quorum-sensing mimics was quantitatively and perhaps qualitatively different after exposure of the seedlings to C_6-HL than after exposure to 3-oxo-C_{12}-HL or to water. This suggests that the secretion of particular AHLs by a bacterium may lead, in turn, to the secretion of different amounts or kinds of signal-mimic compounds by the host, with

the consequent disruption of certain aspects of quorum-sensing regulation in the associated bacterium.

Although we do not yet understand why *M. truncatula* responds to AHLs as it does, we do expect that responses to AHLs will prove to be a sophisticated and relevant part of the long-evolved adaptations of eukaryotic hosts to interaction with bacteria. In part, AHLs may act as virulence factors, eliciting responses like the tissue-damaging inflammation seen in mice (10). In this regard, however, it is pertinent to note that exposure of *M. truncatula* roots to AHLs, even in localized droplets at high concentration, did not result in the development of visible tissue browning or necrosis (Fig. 1), symptoms of the apoptotic hypersensitive response commonly seen in plants responding to pathogenic microbes (25). Alternatively, eukaryotes may have evolved to take advantage of the fact that bacteria must produce AHLs to coordinate their attack on the host. AHLs may thus serve eukaryotic hosts with important quantitative and qualitative information about the presence of certain bacteria that sets in motion an extensive and appropriate set of responses.

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