Long- and Short-Chain Plant-Produced Bacterial *N*-Acyl-Homoserine Lactones Become Components of Phyllosphere, Rhizosphere, and Soil

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Two *N*-acyl-homoserine lactone (acyl-HSL) synthase genes, *lasI* from *Pseudomonas aeruginosa* and *yenI* from *Yersinia enterocolitica*, were introduced into tobacco, individually and in combination. Liquid chromatograph–tandem mass spectrometry and thin-layer chromatography confirmed products of *lasI* and *yenI* activity in single and cotransformants. Cotransformants expressing plastid-localized *LasI* and *YenI* synthases produced the major acyl-HSLs for each synthase in all tissues tested. Total acyl-HSL signals accumulated in leaf tissue up to 3 pmol/mg of fresh weight, half as much in stem tissue, and approximately 10-fold less in root tissues. Acyl-HSLs were present in aqueous leaf washes from greenhouse-grown transgenic plants. Transgenic lines grown for 14 days under axenic conditions produced detectable levels of acyl-HSLs in root exudates. Ethyl acetate extractions of rhizosphere and nonrhizosphere soil from transgenically grown plants contained active acyl-HSLs, whereas plant-free soil or rhizosphere and nonrhizosphere soil from wild-type plants lacked detectable amounts of acyl-HSLs. This work shows that bioactive acyl-HSLs are exuded from leaves and roots and accumulate in the phytosphere of plants engineered to produce acyl-HSLs. These data further suggest that plants that are bioengineered to synthesize acyl-HSLs can foster beneficial plant–bacteria communications or deter deleterious interactions. Therefore, it is feasible to use bioengineered plants to supplement soils with specific acyl-HSLs to modulate bacterial phenotypes and plant-associated bacterial community structures.

Additional keywords: quorum sensing.

The physiology of many bacteria is controlled and modulated in response to population density by producing and monitoring the accumulation of self-produced signal molecules in a process known as quorum sensing (Fuqua et al. 2001; Newton

and Fray 2004; Taga and Bassler 2003). Over 50 species of gram-negative bacteria have been shown to produce and respond to quorum-sensing *N*-acyl-homoserine lactones (acyl-HSLs) signal molecules (Eberhard et al. 1981; Miller and Bassler 2001; Nealson 1977; Whitehead et al. 2001; Winzer and Williams 2001). Quorum-sensing signaling has enhanced our understanding of bacterial survival strategies in nature.

Acyl-homoserine lactone-specific quorum sensing in gram-negative bacteria generally involves two protein components, a signal synthase (LuxI homolog) that catalyzes the synthesis of acyl-HSLs signals and an acyl-HSL-responsive transcriptional factor (LuxR homolog). As the local bacterial population density increases, the acyl-HSLs accumulate to physiologically relevant threshold concentrations and interact with the cognate transcription factor (Whitehead et al. 2001). In most cases, signal-bound LuxR-type proteins activate the regulated target genes (Stevens and Greenberg 1997), whereas others function as transcriptional repressors in a ligand-free state repress (Minogue et al. 2002). The overall effect is the coordinated transcription of specific gene systems in response to a bacterial quorum. These acyl-HSL-regulated gene systems are diverse and include a range of different virulence functions with roles in plant and animal diseases (Cha et al. 1998; Loh et al. 2002; Pierson et al. 1999; Ramey et al. 2004; von Bodman et al. 2003; Whitehead et al. 2002; Winas et al. 1999; Zheng et al. 2003) as well as beneficial activities associated with rhizosphere colonization by plant-growth-promoting bacteria (Lugtenberg et al. 2001; Savka et al. 2002). Different LuxI homolog proteins catalyze the synthesis of a range of specific acyl-HSLs (Eberl 1999). Acyl-HSLs are characterized by a homoserine lactone moiety ligated to an acyl side chain. The specificity derives from the length of the acyl side chain ranging from 4 to 18 carbons, substitution at the C3 position, and unsaturations within the acyl chain (Fuqua and Winans 1999; Gonzalez and Marketon 2003; Laue et al. 2000). Acyl-HSLs are characterized as long-chain or short-chain acyl-HSLs depending on whether their acyl moiety consists of >8 or ≤8 carbons, respectively (Fig. 1). The crystal structure of the *EsaI* and *LasI* signal synthases have been solved (Gould et al. 2004; Watson et al. 2002).

The concept of bioengineering a plant host to produce acyl-HSLs was demonstrated previously by expressing the *yenI* gene from *Yersinia enterocolitica* in tobacco and potato (Fray

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et al. 1999; Toth et al. 2004) and *exlI* from *Erwinia carotovora* (Mae et al. 2001) in tobacco. These studies showed that expressing the acyl-HSL synthase genes from the plant nuclear chromosome and targeting to the chloroplasts promotes the efficient acyl-HSL synthesis. The constitutive expression of *yenI* in tobacco and potato leads to the endogenous accumulation of the major acyl-HSLs for YenI synthase, namely *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) and *N*-hexanoyl-L-homoserine lactone (C6-HSL) (Fray et al. 1999; Toth et al. 2004). The signal molecules 3-oxo-C6-HSL and C6-HSL accumulated to levels sufficient to activate pigment production and luminescence in two different biosensor strains. This suggests that plant-produced acyl-HSLs, such as 3-oxo-C6-HSL and C6-HSL, are freely diffusible across the plastid and plasma membranes. Acyl-HSLs produced by indigenous bacteria of the tomato rhizosphere activate acyl-HSL reporter genes in introduced signal-deficient bacteria even some distance from the acyl-HSL-producing cells (Steidle et al. 2001). This indicates that acyl-HSLs diffuse within the rhizosphere capable of modulating the ecology of rhizosphere bacterial populations (Pierson et al. 1998).

The cultivation of beneficial plant-growth-promoting or biocontrol bacteria, or the selective expression of useful genes in the plant environment, is of significant biotechnological interest (Lugtenberg et al. 2001; Savka et al. 2002). Acyl-HSL signal molecules produced by bioengineered plants may represent an approach to improve the ability of plants to communicate with and regulate beneficial genes in introduced bacteria. In this report, we show that plants expressing the LasI and YenI signal synthases in the chloroplast produce long- and short-chain acyl-HSLs that are readily detectable in the rhizosphere and phyllosphere of the transgenic plants. Moreover, we show that plant-produced acyl-HSLs become an active component of rhizosphere and nonrhizosphere soil.

Fig. 1. Structures of short- and long-chain *N*-acyl-homoserine lactones. Example of **A**, a short-chain (*N*-(3-oxohexanoyl)-L-homoserine lactone) and **B**, long-chain (*N*-(oxododecanoyl)-L-homoserine lactone) *N*-acyl-homoserine lactones.

Table 1. *N*-acyl-homoserine lactone biosensor strains

Bacterium	Receptor	Cognate signal ^z	Source
<i>Agrobacterium tumefaciens</i> NTL4 (pZLR4)	TraR (<i>A. tumefaciens</i>)	3-oxo-C8-HSL	Shaw et al. 1997
<i>Chromobacterium violaceum</i> CV026	CviR (<i>C. violaceum</i>)	C6-HSL (activation) C10-HSL (inhibition)	McClellan et al. 1997
<i>Escherichia coli</i> JM109 p(SB401)	LuxR (<i>Vibrio fischeri</i>)	3-oxo-C6-HSL	Winson et al. 1998
p(SB536)	AhyR (<i>Aeromonas hydrophila</i>)	C4-HSL	Swift et al. 1997
p(SB1075)	LasR (<i>Pseudomonas aeruginosa</i>)	3-oxo-C12-HSL	Winson et al. 1998

^z C4-HSL = *N*-butanoyl-L-homoserine lactone, C6-HSL = *N*-hexanoyl-L-homoserine lactone, 3-oxo-C6-HSL = *N*-(3-oxohexanoyl)-L-homoserine lactone, 3-oxo-C8-HSL = *N*-(3-oxooctanoyl)-L-homoserine lactone, C10-HSL = *N*-(decanoyl)-L-homoserine lactone, 3-oxo-C12-HSL = *N*-(oxododecanoyl)-L-homoserine lactone.

RESULTS

YenI- and lasI-based expression vectors and YenI and LasI transformants.

We used the gene construct pDHERBYIC for plant expression of *yenI* as described previously (Fray et al. 1999). The construct for plant expression of *lasI* is identical to pDHERBYIC, except that the *yenI* coding sequence was replaced with *lasI*. Tobacco transformants of single-gene transformants, containing either *yenI* or *lasI*, were carried to first generation seed (R₁) production. In this study, we used transformed plant line YenI-5, which expresses only *yenI* (Fray et al. 1999), and transgenic plant lines LasI-G, LasI-1, and LasI-5, which express only *lasI* (Fray et al. 1999; unpublished data).

YenI/LasI coexpressing transgenic plants.

In this study, we developed tobacco cotransformants that were transformed with both constructs to express *yenI* and *lasI* genes. In all, 67 independent cotransformants were regenerated and rooted under kanamycin (Km) selection. Antibiotic-resistant cotransformants grown in tissue culture were screened to identify transgenic individuals that accumulated acyl-HSLs in aqueous leaf-cell extractions consistent with the biosynthetic activity of both genes. A representative drop-diffusion assay of 19 cotransformants is shown in Figure 2A. Approximately 20% of the antibiotic-resistant transformants produced a strong response in *Chromobacterium violaceum* biosensor CV026 (McClellan et al. 1997). This biosensor responds preferentially to C6-HSL, but also to 3-oxo-C6-HSL (Tables 1 and 2). A second screen involved the use of two additional acyl-HSL bioluminescence reporter strains, JM109 (pSB401) and JM109 (pSB1075) (Winson et al. 1998). *Escherichia coli* JM109 (pSB401) expresses a native LuxR and responds optimally to 3-oxo-C6-HSL. *E. coli* JM109 (pSB1075) expresses a native LasR with specificity for *N*-(oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Tables 1 and 2; Fig. 1). Six independent, strong violacein-inducing cotransformed transgenic tobacco lines induced light production in the JM109 (pSB401) and JM109 (pSB1075) biosensors measured in relative light units (RLU) (Fig. 2B). Leaf tissue from wild-type tobacco produced background levels of bioluminescence in the biosensor strains, whereas the transgenic lines increased bioluminescence significantly above background indicative of effective YenI and LasI coexpression (Fig. 2B). For estimation of the amount of acyl-HSL present in various plant tissues, we standardized the assay by supplementing wild-type tobacco extracts with various concentrations of synthetic 3-oxo-C6-HSL for JM109 (pSB401) and 3-oxo-C12-HSL for JM109 (pSB1075) as described previously (Winson et al. 1998). Fresh leaf tissue of the cotransformed tobacco plants accumulated up to 50 pM each of 3-oxo-C6-HSL and 3-oxo-C12-HSL (Fig. 2B). Leaf tissue of wild-type tobacco gave only background levels of luminescence with biosensor strains.

LC-MS analysis of LasI activity.

The presence of 3-oxo-C6- and C6-HSL in tobacco plants transformed with pDHERBYIC was confirmed previously by liquid chromatography–mass spectrometry (LC-MS) (Fray et al. 1999). In this study, we used a more sensitive LC-tandem MS (MS-MS) approach to analyze extracts from two plants transformed with the *lasI* gene to confirm that the transgenic

plants exclusively produce the expected 3-oxo-C12-HSL (Fig. 3). This method was sufficiently sensitive to show differences in the level of expression between two transformed lines, and that one of these plants (the higher signal producer of the two) contained trace amount of *N*-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL), a long-chain acyl-HSL whose synthesis had not been anticipated. Using this method, we also

Fig. 2. Identification of cotransformants that synthesize *N*-acyl-homoserine lactones (acyl-HSLs). **A**, Detection of acyl-HSLs in aqueous cell extracts of 19 regenerates. Leaf extracts from 19 independent antibiotic-resistant regenerant plants that were cotransformed with *yenI* and *lasI* gene constructs were assayed using a drop-diffusion assay with *Chromobacterium violaceum* (biosensor strain CV026). Aqueous extracts from leaf tissues of 19 R_0 transgenic plants were prepared and 10- μ l volumes were spotted onto the surface of a CV026-seeded plate. Abbreviations are extracts from 1 to 19 independent cotransformed regenerant plants; C-, wild-type plant; C+, *C. violaceum* wild-type strain culture supernatant extracts at 1 $\times$, 0.1 $\times$, and 0.01 $\times$ (right to left). Other abbreviations: Std = standard of purified *N*-(3-oxohexanoyl)-L-homoserine lactone in ethyl acetate; EA = 10 μ l of EA solvent. **B**, Detection of acyl-HSLs in ethyl acetate fractions of cell extracts from six selected cotransformants. Ethyl acetate samples (20- μ l volumes) were assayed with biosensors JM109 (pSB401) (gray bars) and JM109 (pSB1075) (black bars). Measurements were made at 30.0 and 44.9% sensitivity in relative light units (RLU) for LuxR- and LasR-based biosensors, respectively, using Turner Designs luminometer TD-20/20. Each plant was sampled in triplicate and RLU measured for a 20-s integration period. Values show RLU average (mean $\pm$ standard error of the mean; $n = 3$). Samples include selected cotransformants 5, 26, 29, 31, 39, and 46; WT = wild-type plant. WT plants gave low basal levels of measurable amounts of RLU consistent with background levels seen in the biosensor due to the leakiness of the *luxI*, *rhlI*, and *lasI* promoters in the bioluminescence biosensor strains used in this work (Burmole et al. 2003; Winson et al. 1998). RLU values of transgenic plants are highly significantly different for both biosensors JM109 (pSB401) ($P < 0.01$) and JM109 (pSB1075) ($P < 0.01$).

Table 2. Strains and plasmids

Strain or plasmid	Relevant genotype and phenotype ^a	Source
Strains		
<i>Agrobacterium tumefaciens</i>		
EHA105	Vir-helper strain; pTiBo542	Hood et al. 1993
NtL4	Ti plasmidless derivative of C58, Tc ^s	Luo et al. 2001
<i>Chromobacterium violaceum</i>		
CV026	Acyl-HSL reporter, <i>cviI</i> ::Tn5 ϕ ylE; Tn5 mutant, CviI ⁻	Swift et al. 1997
<i>Escherichia coli</i>		
DH5 α	<i>supE44 lacU169 hsdR17 recA1 endA1 gryA96 thi-1 relA-1</i>	Sambrook et al. 1989
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB) F'</i> [<i>traD36 proAB+ lacIq lacZ M15</i>]	Yanisch-Perron et al. 1985
Plasmids		
PBDHERBYasI	Chloroplast targeted LasI, Km ^r	This work
PBDHERBYIC	Chloroplast targeted YenI, Km ^r	Fray et al. 1999
pSB401	<i>luxR+</i> <i>PluxI-luxCDABE</i> Tc ^r p15A ori	Winson et al. 1998
pSB536	<i>ahyR+</i> <i>PahyI-luxCDABE</i> Amp ^r in pAHP13	Swift et al. 1997
pSB1075	<i>lasR+</i> <i>PlasI'-luxCDABE</i> Amp ^r ColE1 ori	Winson et al. 1998
pZLR4	<i>traR+</i> <i>PtraG::lacZ</i> , Gm ^r	Cha et al. 1998

^a Tc^s and Tc^r = tetracycline susceptible and resistant, respectively; Acyl-HSL = *N*-acyl-homoserine lactone; Km^r = kanamycin resistant; Amp^r = ampicillin resistant; Gm^r = gentamicin resistant.

were able to confirm that the double transformant 33 contained trace amounts of an unexpected 3-oxo-C14-HSL, in addition to the expected 3-oxo-C6- and 3-oxo-C12-HSLs. We also showed that the transgenic line 33, which had been cotransformed with both *yenI* and *lasI* transgenes, produced, in addition to the expected acyl-HSLs, trace amounts of *N*-(3-hydroxy-hexanoyl)-L-homoserine lactone (3-hydroxyl-C6-HSL) (data not shown).

Selection of YenI/LasI cotransformants.

We selected four independent cotransformant R₀ lines numbered 26, 31, 33, and 41 and single transformants YenI-5 and LasI-G for genomic DNA extraction to validate the presence of the transgenes by polymerase chain reaction (PCR). The PCR reactions used either synthetic oligonucleotides specific for the kanamycin resistance gene *nptII* or primers specific for *yenI* and *lasI* (Table 3). A negative control consisted of one primer within the *nptII* gene and the second outside the T-DNA region

Fig. 3. Detection of *N*-(oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) by thin-layer chromatography. Ethyl acetate extracts from aqueous cell macerations were prepared from R₁ cotransformant line 33 and from two *N*-acyl-homoserine lactone (acyl-HSL)-positive descendents of LasI-G (plants 1 and 5). Ethyl acetate samples (10-μl volumes) were assayed with sensor JM109 (pSB1075). Long chain 3-oxo- standards are present in lane O on the left. All three plant extracts contain an acyl-HSL species with the mobility characteristics of 3-oxo-C12 HSL; the presence of 3-oxo-12-HSL in these extracts was confirmed by liquid chromatography-tandem mass spectrometry (not shown).

(Table 3). Transgenes were verified by yielding the expected PCR amplicons from genomic DNA of the four independent cotransformant lines (26,31,33 and 41) and the single transformants YenI-5 and LasI-G (data not shown). No amplicons were detected in reactions with genomic DNA obtained from the untransformed control plants (data not shown).

Presence of short- and long-chain acyl-HSL in leaf extracts of cotransformants.

To characterize acyl-HSL accumulation in cotransformant lines 26, 31, 33, and 41, EtAc fractions of leaf extracts from selfed kanamycin-resistance R₀ progeny were assayed using a different bioassay method (Fig. 4). A LasR-based bioluminescence reporter assay gave between 5 and 39 times higher RLU values than tissue of untransformed wild-type plants. This is consistent with bioluminescent measurements from samples of transformant LasI-G, which contains only *lasI* (Fig. 4). Tissue extracts from the LasI and YenI cotransformants gave 606- and 1,865-fold higher luminescence responses in a LuxR-based biosensor strain. These values are similar to those obtained for the single YenI transformant, YenI-5 (Fig. 4). Thus, these data show that cotransformant transgenic lines which express both *yenI* and *lasI* produce biologically active short- and long-chain signals as anticipated.

Stimulation of TraR-based β-galactosidase.

Twelve-day-old R₁ seedlings from *yenI* and *lasI* transformants and cotransformant line 33, when placed on the surface of agar medium seeded with the TraR-based acyl-HSL biosensor, activated the expression of traG-β-galactosidase reporter function (Farrand et al. 2002) (Fig. 5). In fact, a positive response was detected from transgenic leaf, stem, and root tissue, which indicates that long- and short-chain signals diffuse to the surface of these specific tissues (Fig. 5A).

Inhibition of CviR-based violacein production in transformants LasI-G and YenI-5 and cotransformant 33.

Acyl-HSLs with dramatically different structures can have a cross-inhibitory effect (McClellan et al. 1997). We used the biosensor strain CV026 to demonstrate both signal-specific activation and inhibition of violacein pigment production. To demonstrate both short- and long-chain signal production in YenI-5 and LasI-G transformants and cotransformant line 33, biosensor strain CV026 combined with a low concentration of C4-HSL were used to visualize both inhibition and activation of pigment synthesis in a single assay. The reporter strain CV026 lacks the ability to synthesize any form of acyl-HSL, but carries a functional copy of the CviR receptor, which is specific for C6-HSL but also will respond to significant levels of the 3-oxo-C6-HSL. The YenI-5 transgenic plant and the cotransformant line 33 activated pigment production to various degrees. Both plants produce the 3-oxo-C6-HSL (Fig. 5B). Interestingly, the activity from plant material of the cotransformed line 33 was slightly less active, pre-

Table 3. Oligonucleotides designed for molecular characterization of transgenic plants producing short- and long-chain *N*-acyl-homoserine lactones

Gene	Primer sequence	Predicted amplicon
<i>yenI</i>	5' TGA TGG CAA AGA AAG CGA CCA 3' (forward) 5' TCG GCC AGG TTT CGA GAG TTT 3' (reverse)	503
<i>lasI</i>	5' CGA AAA GGC TGG GAC GTT AGT 3' (forward) 5' GCT CGA TGC CGA TCT TCA GGT 3' (reverse)	427
<i>nptII</i>		
Positive control	5' TGA ATG AAC TGC AGG ACG AGG 3' (forward) 5' GTC ATA GCC GAA TAG CCT C 3' (reverse)	612
Negative control	5' CGC TCT TTT CTC TTA GGT TTA 3' (forward) 5' GTC ATA GCC GAA TAG CCT C 3' (reverse)	469

sumably because of the concomitant inhibitory effect of the coproduced LasI-specific long-chain signal, as reported previously (McClean et al. 1997). This prediction is confirmed by adding leaf extract from LasI-G transformant and by adding pure synthetic 3-oxo-C12-HSL signal which clearly shows the inhibitory effect on violacein (Fig. 5B). Extract from wild-type leaf tissue did not activate or inhibit pigment synthesis (Fig. 5B).

Accumulation of *N*-acyl-homoserine lactones in tissues of tobacco.

To quantify the relative amounts of acyl-HSLs present in different tissues, we evaluated ethyl acetate extracts from leaf, stem, and root tissues of plants grown in tissue culture. Extracts from transgenic lines showed the presence of detectable concentrations of acyl-HSLs in leaf, stem, and root tissue using the TraR-based *Agrobacterium tumefaciens* biosensor strain NTL4 (pZLR4) (Tables 1 and 2; Fig. 6). For estimation of the amounts of total acyl-HSLs present in leaf, stem, and root tissues, known amounts of 3-oxo-C6-HSL were assayed alongside tissue extracts as described (Ravn et al. 2001). Based on these assays, lines 26, 31, and 33 contained in excess of 100 pmol of total acyl-HSLs per milligram of tissue (fresh weight), whereas stems and roots contained from 10 to 50 pmol (Fig. 6). As expected, wild-type tissues did not induce a positive reaction in the TraR-based biosensor assay (Fig. 6). To evaluate the accumulation of acyl-HSLs in soil-grown tobacco, leaf tissue from 20-week-old greenhouse-grown plants were harvested and assayed. The individually transformed lines YenI-5 and LasI-G and the cotransformed lines 26 and 33 induced significant bioluminescence in the reporter assay compared with wild-type plants (Table 4). As-

Fig. 4. Detection of *N*-acyl-homoserine lactones (acyl-HSL) in *R*₁ cotransformants. Ethyl acetate fractions from tissue extracts were prepared from the wild type (WT); selected *R*₁ cotransformants 26, 31, 33, and 41; and single transformants LasI-G and YenI-5. Ethyl acetate samples (20- μ l volumes) were assayed with sensors JM109 (pSB401) (gray bars) and JM109 (pSB1075) (black bars). Each plant was sampled in triplicate. Luminescent levels are expressed in relative light units (RLU). RLU values of transgenic plants are significantly enhanced over the basal levels for both biosensors JM109 (pSB401) ($P < 0.01$) and JM109 (pSB1075) ($P < 0.01$).

says using the LuxR-based biosensor JM109 (pSB401) yielded bioluminescence values greater than 250-fold compared with the wild-type background levels (Table 4). The assays using the LasR biosensor JM109 (pSB1075) increased

Fig. 5. Detection of short- and long-chain *N*-acyl-homoserine lactones (acyl-HSL) signals in transformant and cotransformant. **A**, Detection of acyl-HSLs on the surface of transformed and cotransformed seedlings. Stimulation of β -galactosidase activity in *Agrobacterium tumefaciens* biosensor strain NTL4 by transformants YenI-5 and LasI-G and cotransformant 33. Wild-type (WT) seedlings show lack of stimulation in NTL4 biosensor. Twelve-day-old *R*₁ seedlings germinated and grown in tissue with antibiotic selection were placed on the surface of agar medium seeded with NTL4 (pZLR4) biosensor strain supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. A blue halo surrounding seedlings indicate exudation of plant-produced acyl-HSLs. **B**, Inhibition and stimulation of violacein production in CV026 with leaf extracts. Short-chain signal C4-HSL (final concentration, 2.42 μ M) together with CV026 was added to a soft agar plate. Inhibition of violacein production is defined by the presence of a white halo around the well in a light purple background. A halo is seen to the right of the arrow (identified by a horizontal black bar), indicating inhibition by LasI-G extract. Stimulation of pigmentation in the biosensor is defined by the presence of a purple halo around the well as evident in transformant YenI-5 and cotransformant 33. Extract from WT leaf tissue did not activate or inhibit pigment synthesis. Positive control well (3-oxo-C12) contains 1.0 μ l of 1 mM pure *N*-(oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL). Negative control well (H₂O) contained a 20- μ l volume of pure water.

80-fold (Table 4). We also used an AhyR-based biosensor with specificity for *N*-butanoyl-L-HSL (C4-HSL) (Tables 1 and 2). Extracts assayed from the transgenic and wild-type lines gave essentially the background level reactions in this particular biosensor (Table 4), indicating that the C4-HSL is absent or only present in insignificant levels.

Presence of *N*-acyl-homoserine lactones in the phyllosphere.

Leaf washes were analyzed based on luminescence induction in the biosensor strains JM109 (pSB401) and JM109 (pSB1075) to determine the levels of acyl-HSLs exuded onto various plant

surfaces of soil-grown transgenic plants. As shown in the summarized data of Figure 7A, levels of acyl-HSLs sufficient to promote reporter activity are detected in leaf washes of the transgenic lines YenI-5 and LasI-G and the cotransformant 26 (Fig. 7A). In contrast, washes from leaves of wild-type plants did not contain detectable amounts of acyl-HSLs (Fig. 7A and B).

Root exudation of *N*-acyl-homoserine lactones.

Cotransformant lines were tested for exudation of acyl-HSLs from roots by growing plants in a hydroponic system. Growth media assayed over a period of 77 days showed that acyl-HSLs diffused into the medium at detectable concentrations using the TraR-based biosensor after 14 days of plant growth in all transgenic lines (data not shown). More significantly, both short- and long-chain acyl-HSLs accumulated in the hydroponic media over a 77-day growth period (Fig. 8). For example, cotransformant line 31 R₁ progeny accumulated approximately 35 nM 3-oxo-C6-HSL and 10 nM 3-oxo-C12-HSL (Table 5). Hydroponic culture supernatants from growth medium of YenI-5 R₁ progeny produced approximately 35 nM acyl-HSLs. However, this concentration presumably was below the threshold required to induce a response in assays using biosensor JM109 (pSB1075). Hydroponic supernatants from the growth medium of LasI-G R₁ progeny produced approximately 5 nM acyl-HSLs; this amount was sufficient for the induction of the LasR-based biosensor system but, as expected, did not induce the LuxR-based strain (Table 5).

Presence of *N*-acyl-homoserine lactones in soils.

We wished to determine whether plant-produced acyl-HSLs accumulate in the soil to concentrations sufficient to influence rhizosphere bacterial populations. We transplanted 3-week-old seedlings to soil saturated with water. Soils were assayed after 8 weeks of plant growth. The acyl-HSLs were extracted from various soil samples and tested in a diffusion-well assay using biosensor NTL4 (pZLR4). All soil samples from transgenic plants contained detectable levels of bioactive acyl-HSLs in both rhizosphere samples, whereas those of the wild-type plant failed to yield a response (Fig. 9A). This indicates that the wild-type extracts lacked authentic acyl-HSLs, acyl-HSL agonists, or mimic substances. Tightly adhering soil samples of cotransformed plant lines 26 and 33 accumulated up to 2 pmol of extractable acyl-HSLs per milligram of soil (Fig. 9A). Acyl-HSLs also were analyzed in loose and tightly associated soils of plants grown to anthesis in the greenhouse. Both types of soil samples associated with transformants YenI-5 and LasI-G and cotransformed lines 26 and 33 produced detectable levels

Fig. 6. Semi-quantification based on TraR-based biosensor of total *N*-acyl-homoserine lactones (acyl-HSLs) in leaf, stem, and root tissues of selected axenically grown transformants and cotransformants. **A**, Total acyl-HSLs in leaf, stem, and root tissues of transformants and cotransformants. Each sample represents an ethyl acetate fraction from 20 mg fresh weight of leaf, stem, and root tissue of cotransformants 26, 31, 33, and 41. Identical ethyl acetate fractions from cell extracts of transformed plants containing *lasI*, LasI-G (L); *yenI*, YenI-5 (Y), and wild type (WT) also were prepared and assayed. **B**, Dose-response curve to estimate the amounts of purified *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) in well-diffusion detection assays based on NTL4 (pZLR4) biosensor (Ravn et al. 2001). Diffusion wells (left to right) contain 10, 50, 100, 500, 0 (ethyl acetate [EA] only), and 1,000 pmol of 3-oxo-C6-HSL. Regression: $y = 4.1216 \log(x) + 10.064$ ($R^2 = 0.994$).

Table 4. Accumulation of *N*-acyl-homoserine lactones in leaves of greenhouse-grown transgenic plants^w

Plant line ^y	RLU produced by <i>Escherichia coli</i> strain JM109 containing acyl-HSL bisensor plasmid: ^x		
	pSB401 (3-oxo-C6-HSL) EtAc	pSB536 (C4-HSL) EtAc	pSB1075 (3-oxo-C12-HSL) EtAc
YenI-5	11,789 a	91 a	235 b
LasI-G	499 d	35 d	8,257 a
26	9,337 b	54 bc	8,281 a
31	4,643 c	70 b	8,306 a
WT-s	34 d	34 d	119 b
None ^z	38 d	46 cd	91 b

^w Supernatants of ethyl acetate (EtAc) extracts (20 µl) were dried in 12-by-50-mm round-bottom tubes; then, 200 µl of a 1/10 dilution of an overnight culture of sensor strain was added. Tubes were incubated for 5 h at 30°C with gentle shaking. Luminescence was measured as relative light units (RLU) with a Luminometer TD-20/20 (Turner Designs). Each determination is the average of three samples followed by standard deviation. Leaf extract per tube was equivalent to 20 mg fresh weight. RLU values of genotypes are significantly different ($P < 0.01$) for biosensors JM109 (pSB401), JM109 (pSB536), and JM109 (pSB1075). In a single column, values followed by a common letter are not significantly different at the 5% level.

^x Cognate *N*-acyl-homoserine lactones (acyl-HSL) for each biosensor strain; 3-oxo-C6-HSL = *N*-(3-oxohexanoyl)-L-homoserine lactone, 3-oxo-C12-HSL = *N*-(oxododecanoyl)-L-homoserine lactone.

^y Abbreviation of plant lines include: *yenI*, YenI-5; *lasI*, LasI-G; *lasI* and *yenI*, 26; wild-type *Nicotiana tabacum* cv. Samsun, WT-s; and None, sensor strain in the absence of extract.

^z Background RLU of biosensor in absence of extract.

of acyl-HSLs (Fig. 9B). Similar soil samples from wild-type plants did not contain extractable acyl-HSLs (Fig. 9).

DISCUSSION

This study shows that plants engineered to express, separately or in combination, the acyl-HSL signal synthase gene *yenI* and *lasI* in plastids produce biologically relevant levels of short- and long-chain acyl-HSLs (Figs. 1, 3, 4, and 5). Interestingly, we

also found that *LasI* signal synthase expressed in the plant background is capable of synthesizing a small amount of 3-oxo-C14-HSL in engineered plants, suggesting that the fidelity of *LasI* protein is less stringent in the plant background or that the plant cell environment provides sufficient levels of the C14-ACP precursor substrate. Acyl-HSLs are present in all major tissues tested. More significantly, QS signals are found in leaf and root washings at biologically relevant levels (Figs. 4, 7, and 8; Table 5). Biologically active concentrations of acyl-HSLs also exuded into both loosely and tightly adhering soil fractions associated with roots of engineered plants (Figs. 8 and 9).

Fig. 7. Detection of *N*-acyl-homoserine lactones (acyl-HSLs) from phyllosphere washes and corresponding leaf extracts. **A**, Presence of acyl-HSL signals in leaf washes. Transformants included YenI-5, YenI; LasI-G, LasI; cotransformant 26, 26; and wild type, WT. Ethyl acetate fractions of aqueous leaf washes were prepared and triplicate samples and 10- μ l volumes were assayed with *Escherichia coli* JM109 (pSB401) (gray bars) or JM109 (pSB1075) (black bars). Tubes were incubated at 30°C for 5 to 6 h with shaking at 100 rpm. Luminescent responses of biosensors in relative light units (RLU) were measured with luminometer TD-20/20 (Turner Designs) at 30.0% sensitivity for JM109 (pSB401) and at 50.1% sensitivity for JM109 (pSB1075). Values show RLU average (mean $\pm$ standard deviation; $n = 3$). RLU values of genotypes are significantly different for both biosensors JM109 (pSB401) ($P = 0.02$) and JM109 (pSB1075) ($P < 0.01$). **B**, Presence of acyl-HSL signals in extracts of corresponding washed leaves. Triplicate samples of 10 μ l volumes of ethyl acetate fraction from aqueous cell extracts were assayed with biosensors JM109 (pSB401) (gray bars) and JM109 (pSB1075) (black bars). RLU values of genotypes are significantly different for both biosensors ($P < 0.01$).

Fig. 8. Accumulation of *N*-acyl-homoserine lactones (acyl-HSLs) in root exudate of transformants and cotransformant. **A**, Accumulation of short-chain acyl-HSLs as root exudates detected with LuxR-based sensor JM109 (pSB401). Plants were grown in liquid Murashige and Skoog medium (MSM) supported on sterile cheesecloth and samples were taken axenically at the times indicated from the wild type (WT), *yenI* (YenI) and *lasI* (LasI) transformants, and *yenI* and *lasI* cotransformant line 33 (33). Aliquots of individual samples were extracted in ethyl acetate and triplicate samples of 20- μ l volumes were assayed. Bioluminescent responses of biosensors in relative light units (RLU) were measured with a luminometer TD-20/20 (Turner Designs). Bioluminescent responses of fresh MSM were 68 ± 12 and 62 ± 5 for LuxR- and LasR-based biosensors, respectively (not shown). Values show RLU average (mean $\pm$ standard deviation; $n = 3$). RLU values of genotypes and time are highly significantly different for biosensor JM109 (pSB401) ($P < 0.01$). **B**, Accumulation of long-chain acyl-HSLs as root exudates detected with LasR-based sensor JM109 (pSB1075). RLU values of genotypes and time are highly significantly different for biosensor JM109 (pSB1075) ($P < 0.01$).

The accumulation of acyl-HSLs by LuxI homologs in heterologous plant backgrounds (Fray et al. 1999; Mae et al. 2001; Toth et al. 2004) suggests that plants synthesize the necessary precursor metabolites for different acyl-HSL synthesis. In our work, plastid-localized YenI and LasI signal synthases supported the synthesis and accumulation of biologically relevant levels of acyl-HSLs in leaf, stem, and root, with the highest accumulation in leaf followed by stem and root tissue (Fig. 6). This accumulation pattern may reflect the greater abundance of plastids (chloroplasts) and the higher activity of key metabolic pathways (particularly fatty acid synthesis) in photosynthetically active tissues. Acyl-HSLs also accumulated in nonphotosynthetic root tissue, suggesting that YenI and LasI have substrates for acyl-HSL synthesis in proplastids or other nonchlorophyllous plastids such as amyloplasts present in root cells. Work by Toth and associates (2004) showed that, in transgenic potato, targeting of YenI to plastids resulted in approximately 10 times more C6-HSL in stem than in nonphotosynthetic tuber tissue. However, root accumulation of acyl-HSLs may be influenced by translocated acyl-HSLs produced in photosynthetically active leaf tissue. Further work is necessary to evaluate whether acyl-HSLs are translocated between plant tissues. We have produced hairy-root cultures from transgenic line YenI-5 plants and have shown that YenI-5 hairy-root cells produce acyl-HSL. Moreover, hairy-root growth media accumulated detectable amounts of signals (R. Scott and M. Savka, *unpublished data*), supporting our observations and those by Toth and associates (2004) that normal root tissue of bioengineered plants produce, accumulate, and exude acyl-HSLs.

This is the first report of the bio-engineering of plants to produce long-chain acyl-HSLs and is also the first detailed report showing exudation of acyl-HSLs onto leaf surfaces (Fig. 7), although short-chain acyl-HSL secretion by roots has been reported (de Angelo-Picard et al. 2004). The QS signals are detected in aqueous washes from the surface of leaves. Although our washing period was less than 60 s to minimize the possibility of signal leaching, we cannot completely discount the possibility that some of the acyl-HSL fractions are the result of ruptured or leaking epidermal cells or trichomes. However, what is clear is that acyl-HSLs accumulate to high levels in leaf tissue (Table 4) and that signals are present on the leaf surface in concentrations sufficient to activate LuxR homolog-based biosensor bacteria (Fig. 7). This suggests that plants engineered to produce specific acyl-HSL may be used to generate and/or modulate the communication channel between the plant and phyllosphere inhabiting

beneficial or pathogenic bacteria. Plant-produced acyl-HSL signals may disrupt the normal discourse of bacterial infection processes and, thus, reduce disease incidence. For example, the leaf blight and Stewart's wilt pathogen, *Pantoea stewartii* subsp. *stewartii*, regulates its major virulence factor using 3-oxo-C6-HSL. Corn engineered to produce EsaR agonistic acyl-HSL signals may lead to premature synthesis of exopolysaccharide production, which is detrimental to the disease process of this vascular pathogen (von Bodman et al. 1998, 2003). We are testing the effect of transgenic maize-produced bioactive levels of 3-oxo-C6-HSL on the ability of *P. stewartii* to cause Stewart's wilt.

Transgenic plants grown in liquid culture media exude bioactive acyl-HSLs from their roots during hydroponic growth (Fig. 8). Acyl-HSLs were detected after 14 days of incubation and continued to accumulate over a 77-day period. It is possible that some of the signals originated from lysed root epidermal or sloughed root cap cells, although we feel that this contribution is minimal because tobacco roots yield few peripheral cap cells (Hawes et al. 2000; Hawes and Pueppke 1986). Thus, our results are a strong indication that transgenic roots exude plant-produced acyl-HSLs into hydroponic medium which, in turn, suggests that root exudates can enrich the rhizosphere with bioactive acyl-HSLs.

Barber and Gunn (1974) reported that organic substances exuded from plant roots accumulate to higher levels in sand than in liquid medium (Barber and Gunn 1974). We tested whether this observation applies to the accumulation of acyl-HSL. Acyl-HSLs were present in soil associated with roots of plant engineered to produce long-, short-, and both long- and short-chain acyl-HSLs in tightly and loosely plant root-associated soils (Fig. 9). Tightly adhering soil samples of cotransformed plant lines 26 and 33 accumulated up to 2 pmol of extractable acyl-HSLs per milligram of soil. These data are consistent with others reported for plant-produced 3-oxo-C6-HSL in the rhizosphere soil containing a dense root system (d' Angelo-Picard et al. 2004), in soil (Burmolle et al. 2003), and in a tomato rhizosphere (Steidle et al. 2001). Our data also suggests that acyl-HSLs can diffuse into the soil to some distance from the root. This is consistent with the work of Steidle and associates (2001), who showed that the natural bacterial community in the tomato rhizosphere is able to activate acyl-HSL sensors on the root surface even when the acyl-HSL-producing community members inhabited a location at some distance from the root.

The sustained accumulation of acyl-HSL in soil and hydroponic supernatants suggest that the synthesis and exudation ex-

Table 5. Accumulation of short- and long-chain *N*-acyl-homoserine lactones (acyl-HSLs) in root cultural supernatants of transgenic plants grown for a 77-day period^w

Plant line	Average RLU ^x		Signal concentration in nM root exudate (approximate) ^{x,y}	
	JM109 (pSB401) (3-oxo-C6-HSL)	JM109 (pSB1075) (3-oxo-C12-HSL)	3-oxo-C6	3-oxo-C12
26	2,434 c	2,832 a	25	10
31	2,841 b	2,253 b	35	10
33	1,653 d	2,102 b	20	10
41	1,647 d	1,987 b	20	5
WT-s	88 e	59 d	0	0
LasI-G	112 e	874 c	0	5
YenI-5	3,252 a	58 d	35	0
None ^z	87 e	47 d	0	0

^w Supernatants of hydroponic fluid (20 µl) from plant cultures after 77 days of heterotrophic growth were analyzed with sensors JM109 (pSB401) and JM109 (pSB1075) for bioluminescence. Relative light units (RLU) values of genotypes are significantly different ($P < 0.01$) for both biosensors JM109 (pSB401) and JM109 (pSB1075). In a single column, values followed by a common letter are not significantly different at the 5% level.

^x Cognate acyl-HSL for each biosensor strain; 3-oxo-C6-HSL = *N*-(3-oxohexanoyl)-L-homoserine lactone, 3-oxo-C12-HSL = *N*-(oxododecanoyl)-L-homoserine lactone.

^y Root exudates in hydroponic supernatants of liquid growth medium in which the root system was submerged during plant growth.

^z Background RLU of biosensor in absence of extract.

ceed their potential degradation levels in the environments, at least under our experimental conditions. The nonenzymatic turnover occurs during laboratory culture as a result of lactonolysis at high pH in response to high temperature and is augmented in signals with electron-withdrawing side chains (Byer et al. 2002; Yates et al. 2002). Recent work by Wang and Leadbetter (2005) shows rapid acyl-HSLs biodegraded in soils sampled from various sites and that this biodegradation is much faster than chemical lactonolysis. A number of bacteria have the ability to enzymatically inactivate acyl-HSLs (Zhang 2003; Zhang and Dong 2004). Enzymatic degradation occurs by hydrolyzing the ester bond of the lactone ring to produce acyl-homoserine (Dong et al. 2000, 2001) or by cleavage of the amide bond between the HSL ring and acyl side chain (Leadbetter and Greenberg 2000).

An effect of acyl-HSL degradation products on plant physiology was reported by Joseph and Phillips (2003), showing that treatment of bean roots with physiologically relevant concentrations of homoserine lactone and homoserine significantly increases stomatal conductance and transpiration of the bean. Transpiration also enhances the availability of mineral nutrients for the growth of plants and root-associated bacteria. It is conceivable that the bacterial production of acyl-HSLs and their degradation to homoserine lactone and homoserine may represent a plant-microbe interaction in which both the plant and root-associated bacteria benefit from the production of QS signals in the rhizosphere (Joseph and Phillips 2003).

The mechanism by which acyl-HSLs are exuded from plant cells is not known. In *P. aeruginosa*, the transport of long-chain acyl-HSL (3-oxo-C12-HSL) into the growth medium requires an efflux pump encoded by mexAB-oprM operon, suggesting that long-chain acyl-HSLs partition into the lipid bilayer unless pumped out of the cell. This is in contrast to the *P. aeruginosa* short-chain signal (C4-HSL), which freely diffuses into and out of the cells (Pearson et al. 1999). In this work, exudation of acyl-HSLs apparently takes advantage of secretion mechanisms already operative in the plant. Implicated in the mechanism of QS is the ability of acyl-HSLs to diffuse freely into and out of the cell across the membrane (Manefield and Turner 2002; Redfield 2002). In order to foster plant-bacteria communication, acyl-HSL signals are required to exude from the plant surface and to become available for the resident plant-associated bacteria. This is exactly what we have observed; bioactive bacterial QS signals are present on the plant surface and acyl-HSLs become components of the soluble plant exudates released into the phylloplane and rhizosphere (Figs. 7 and 8) and become components of the rhizosphere soil (Fig. 8).

In conclusion, we successfully demonstrated that transgenic plants express the bacterial YenI and LasI proteins, individually or in combination within plant cell plastids. We further showed that these heterologous proteins are functional and capable of producing biologically relevant levels of acyl-HSLs. LC followed by MS-MS and thin-layer chromatography confirmed the expected signals products of YenI and LasI activity in tobacco. Transgenic plants expressing *lasI* also produced detectable levels of 3-oxo-C14-HSL, which was unexpected. Bioassays using the reporter strains *A. tumefaciens* NTL4 (pZLR4), *C. violaceum* CVO26, and three *E. coli* luminescent-based reporters showed the presence of plant-produced acyl-HSLs in cell extracts of leaf, stem, and root tissues. These assays also showed that signals become a soluble component of the phyllosphere and rhizosphere. In addition, we showed that the 3-oxo-C12-HSL-produced signal inhibits or reduces the stimulatory effect of 3-oxo-C6-HSL short-chain signal in a CviR-based bioassay. Accordingly, this work demonstrates the feasibility of designing acyl-HSL-specific plant-bacteria biosystems

that can enhance beneficial plant-microbe interactions or inhibit harmful interactions.

MATERIALS AND METHODS

Bacterial strains and plasmids.

E. coli strains were grown in Luria-Bertani (LB) medium at 37°C, whereas *A. tumefaciens* binary transformation strains and the acyl-HSL biosensor strain *C. violaceum* CVO26 were grown at 26°C in LB and tryptone-yeast extract (TYE) media, respectively. *A. tumefaciens* acyl-HSL biosensor strain NTL4 (pZLR4) was grown in AB minimal medium salts (Chilton et al. 1974) supplemented with 0.1% glucose and 0.01% yeast extract. All media were solidified with 16 g of Bacto Agar (Difco Laboratories, Detroit) per liter. For plasmid propagation and selection of transformations, media was supplemented with the antibiotics ampicillin (100 µg/ml), gentamicin (30 µg/ml), kanamycin (50 µg/ml), and tetracycline (20 µg/ml) for *A. tumefaciens* and *E. coli*. For *C. violaceum*, tetracycline was used at 5 µg/ml. For *A.*

Fig. 9. Detection of *N*-acyl-homoserine lactones (acyl-HSL) signals in rhizosphere and nonrhizosphere of **A**, axenically and **B**, greenhouse-grown acyl-HSL-producing plants. **A**, Detection of acyl-HSL signals from tightly adhered rhizosphere soil and loosely adhered rhizosphere of axenically grown acyl-HSL-producing plants. Rhizosphere soil was extracted in 4:1 ethyl acetate/soil (vol/vol). Soil from transformants LasI-G, (Las) and YenI-5 (Yen) and cotransformants: 26, 31, 33, and 41. Each well sample represents 40 mg of loosely adhering (left well) or tightly adhering (right well) soil. Negative controls include extracts of plant-free soil (PFS) and wild-type tobacco cv. Samsun (WT-s) and cv. Xanthi (WT-x). Standards include purified *N*-(3-oxohexanoyl)-L-homoserine lactone in ethyl acetate in the amounts 10, 50, 0, and 100 pmol (left to right). **B**, Acyl-HSL signals detected in loosely adhering (top well) and tightly-adhering (bottom well) soils of greenhouse-grown acyl-HSL-producing plants. Negative controls include ethyl acetate solvent only (EA) and ethyl acetate fractions of plant-free soil (PFS) and of loosely adhering (top well) and closely adhering (bottom well) soils in which wild-type cv. Xanthi (WT-x) tobacco were grown. Data shown in panels A and B are from a representative experiment that was independently replicated three times.

tumefaciens NTL4 (pZLR4), 5-bromo-4chloro-3-indolyl- β -D-galactopyranoside (X-gal) was used at 40 μ g/ml in diffusion-well assays (see below). Bacterial strains and plasmids used in this study are described in Tables 1 and 2.

Chemicals.

Purified standards of *N*-acyl-homoserine lactones were purchased from Quorum Sciences, Inc. (Coralville, IA, U.S.A.). X-gal was purchased from Stratagene (La Jolla, CA, U.S.A.). Bacto agar was purchased from Difco Laboratories. All other chemicals were purchased from Sigma-Aldrich (St. Louis).

Construction of T-DNA vectors for plant expression of *yenI* and *lasI*.

Binary plant transformation vectors were constructed to express *yenI* from *Y. enterocolitica* and *lasI* from *P. aeruginosa* in tobacco. Construction of the *yenI* plant expression vector pBDHERBYIC was described previously (Fray et al. 1999; Toth et al. 2004). The *lasI* cassette was constructed as follows: the *lasI* sequence was PCR amplified using the oligonucleotides 5'*lasI* GGATCCGCATGCTCGTACAAATTGGTCCG and 3'*lasI* GGAGCATGCTCTAGATCATGAAACCGCCAGT CGC. This introduces *SphI* sites at each end of the PCR product. The *SphI* site in 5'*lasI* encodes the initiating methionine (ATG) and overlaps the second codon. As a result, the second amino acid of the mature LasI protein is changed from isoleucine to leucine; however, we show in bacteria that the ability to synthesize 3-oxo-C12-HSL was unaffected. The *SphI lasI* fragment was ligated into pBDHERBYI (Fray et al. 1999) from which the *yenI* coding sequence had been removed by digestion with *SphI*. This resulted in a translational fusion between *lasI* and the petunia *rbsS* ribulose biphosphate small-subunit chloroplast-targeting sequence under the control of CaMV35S promoter and the alpha mosaic virus translational enhancer sequence (Fray et al. 1999). After sequencing to confirm the correct transgene structure, the *lasI* expression cassette was released as a *PvuII* cut fragment and ligated into pBIN19 (Bevan 1984) previously cut with *SmaI*. This gave the plant binary transformation vector pBDHERBlasI that was introduced into *A. tumefaciens* vir-helper strain EHA105 by electroporation using a Bio-Rad Micropulser. The resulting transformants were screened for kanamycin resistance on LB medium. The *yenI* binary vector, pBDHERBYIC, was transferred similarly to EHA105 and the presence of the *yenI* and *lasI* T-DNA vector in each EAH105 strain was confirmed by isolation of total plasmid DNA followed by endonuclease restriction analysis. The T-DNA vector carrying the *yenI* gene is designated pBDHERBYIC and the vector carrying *lasI* is named pBDHERBlasI.

Generation of transgenic plants and growth conditions.

Each binary strain, containing either pBDHERBYIC (*yenI* construct) or pBDHERBlasI (*lasI* construct), was used for transformation of tobacco leaf tissue according to our protocol (Savka and Binns 2000). For cotransformation of *yenI* and *lasI*, overnight cultures of each strain were harvested and diluted 10-fold in LB medium. Equal volumes of each diluted strain then were mixed and used for transformation.

Identification

by LC-MS-MS and thin-layer chromatography.

An Agilent 1100 series vacuum degasser, autosampler, and binary capillary LC pump was used in line with a Waters Micromass Quattro Ultima Mass spectrometer. Injections (5- μ l) were applied to a C18 column (Exsil Pure MS, 5 μ m 250 $\times$ 2.1 mm; Alltech Associates Ltd., Lancashire, U.K.). The elution procedure was conducted using an isocratic profile of acetonitrile-water (60:40, vol/vol) for 15 min followed by a linear gradient from 60 to 100% acetonitrile in water over 5 min. After 10 min at 100% acetonitrile, the column was re-equilibrated. The LC system was coupled directly to the mass spectrometer probe, without splitting the 0.200 ml/min flow. Standards of the unsubstituted, 3-oxo-, and 3-hydroxy series at 1 μ g/ml were used throughout. For each standard, the [M+H]⁺ parent ions were selected using the minimum cone voltage (35 V) (Table 6).

The source and desolvation temperatures were 125 and 350°C, respectively. Daughter ions of each of the anticipated acyl-HSLs were monitored using positive ion electrospray mode throughout each LC experiment. During each 1-s scan, the collision cell was ramped between 10 and 25 V and the helium CID gas was set at 5 $\times$ 10⁻³ mbar. The instrument had been tuned such that the resolution was 0.7 Da wide at half height. Each of the nine scan functions monitored daughter ions between 85 to 350 Da. Acyl-HSL standards were identified and confirmed by producing extracted ion chromatograms of the 101.6-Da moiety and comparing both the elution time and the spectra from any peaks obtained with those of the standards. Thin-layer chromatography was performed as described by Yates and associates (2002).

Isolation of total DNA and PCR analysis.

Chromosomal DNA from selected plant transformants was isolated using a DNeasy plant mini kit (Qiagen, Hilden, Germany). The *yenI*, *lasI*, *nptII*, and T-DNA vector nucleic sequences were amplified with specific primers (Table 3) using Clontech touchdown PCR genomic DNA kit (Clontech, Palo Alto, CA, U.S.A.). Table 3 contains the sequences of the specific primers for *yenI*, *lasI*, *nptII*, and T-DNA vector and the predicted amplicon sizes.

N-acyl-homoserine-lactone biosensor strains.

Presence of acyl-HSLs in sample extracts was determined using three different reporter bacteria in which a detectable phenotype depends upon exogenous acyl-HSLs (Brelles-Marino and Bedmar 2001; Camara et al. 1998; Cha et al 1998; Farrand et al 2002; McClean et al. 1997; Shaw et al. 1997). A diffusion assay using *C. violaceum* CV026 was used for the initial screen of 67 independent transgenic regenerates (McClean et al. 1997). In *C. violaceum* CV026, the LuxR homolog, CviR, regulates the production of a purple pigment with exogenous acyl-HSLs (McClean et al. 1997). A well-diffusion assay to semiquantify acyl-HSLs in samples employed the versatile *A. tumefaciens* NTL4 (pZLR4) (Farrand et al. 2002). *A. tumefaciens* NTL4 (pZLR4) lacks *traI* and, thus, does not produce acyl-HSLs. Plasmid pZLR4 contains the *lacZ* structural gene fused to *traG* that features a TraR-dependent promoter. In the

Table 6. *N*-acyl-homoserine lactones (acyl-HSL) [M+H]⁺ parent ions used in liquid chromatography–tandem mass spectrometry analysis

Series	[M+H] ⁺ parent ions					
	C4	C6	C8	C10	C12	C14
Unsubstituted acyl-HSLs	172	200	228	256	284	312
3-Oxo-HSLs	186	214	242	270	298	326
3-Hydroxyl-HSLs	188	216	244	272	300	328

absence of detectable acyl-HSLs via TraR, sensor NTL4 (pZLR4) does not produce β -galactosidase and remains white on medium containing the chromogenic substrate, X-Gal (Farrand et al. 2002). However, when exposed to optimum amounts of bioactive acyl-HSL signals, the strain produces β -galactosidase and forms blue halos surrounding the bacterial colony when grown on medium containing the chromogenic substrate (Cha et al 1998; Farrand et al. 2002; Shaw et al 1997).

The additional acyl-HSL biosensor strains used in this work are *E. coli* based (Tables 1 and 2) and contain plasmid pSB401, pSB1075, or pSB536 (Winson et al. 1998). These reporters in strain JM109 employ different R-protein acyl-HSL receptor proteins. For example, JM109 (pSB401) contains the LuxR receptor and will produce light in response to exogenous acyl-HSLs via the activation of the luxCDABE operon. JM109 (pSB401) detects picomolar amounts of *N*-(3-oxo-hexanoyl)-L-HSL and is responsive to varying degrees to acyl-HSLs with side chains ranging from 4 to 12 carbons in length irrespective of the substitution at the 3 position (Camara et al. 1998). Related JM109 *E. coli*-based sensor strains containing plasmids pSB1075 or pSB536 utilize LasR and AhvR receptor proteins designed to activate the lux reporter operon in response to certain acyl-HSLs. Acyl-HSL sensor strains and their cognate signals are listed in Table 1.

***N*-acyl-homoserine lactone extractions and detection assays.**

Plant tissue samples. Cell extractions of root, stem, and leaf tissues were prepared from axenic plants and greenhouse-grown plants. Tissue extracts were prepared by macerating between 300 and 600 mg of tissue. In all cases, aqueous supernatants were recovered after 5 min of centrifugation at 12,000 rpm. The sample was diluted with sterile, purified water to adjust the total aqueous supernatant recovered to working concentrations of 1 μ l/ml fresh weight. Aqueous supernatants, unless otherwise noted, were extracted with equal volumes of acidified ethyl acetate (EtAc).

Aqueous supernatants, synthetic acyl-HSLs, or EtAc extractions were screened for acyl-HSLs in drop-diffusion and well-diffusion assays. For drop-diffusion assays, an up to 20- μ l volume of assay sample was applied onto the surface of the seed agar medium. In well-diffusion assays, samples were added into wells of an agar plate seeded with a specific biosensor strain. The agar plates containing *A. tumefaciens* NTL4 (pZLR4) were prepared as described previously (Farrand et al. 2002). For whole seedling assays, 3-week-old seedlings were grown in tissue culture medium in the presence of kanamycin and subsequently transferred to the surface of the agar medium seeded with biosensor strain NTL4 and X-gal.

CviR-based biosensor activation and inhibition assays. *C. violaceum* biosensor strain CV026 (McClean et al. 1997) was grown overnight in TYE medium, diluted 10-fold, and grown for an additional 5 to 6 h. Seeded agar plates were prepared by mixing 60 ml of diluted CV026 culture with equal volumes of fresh TYE medium and 2 $\times$ Bacto agar, the later two maintained at 50°C. The signal receptor in CV026, CviR, is 140-fold more sensitive to stimulation by short-chain, C6-HSL compared with inhibition by long-chain signal 3-oxo-C12 (McClean et al. 1997). EtAc extracts from aqueous tissue macerations were prepared from wild-type R₁ cotransformant 33 and single transformants LasI-G and YenI-5. Each well illustrated in Figure 5B represents 1.5 g fresh weight of tissue extracted twice in EtAc, dried and resuspended in 20 μ l of water. By using a minimal concentration of C4-HSL, both inhibition and activation of pigment synthesis can be visualized in a single assay. For these inhibitory or activation assays, biosensor *C. violaceum* CV026 soft agar medium (10.67% agar) was

supplemented with C4-HSL at a final concentration of 2.4 μ M and poured into a 245-mm² NUNC bioassay dish. In well-diffusion assays, 4-mm-diameter wells were punched into the bacterial-seeded agar with a sterile cork borer. Up to 30 μ l of solution samples were added into each well. Plates were incubated overnight at 28°C.

Phyllosphere samples. To prepare samples from the leaf surface, the adaxial leaf surface of greenhouse-grown plants were rinsed with 50 μ l of sterile purified water. The 50- μ l rinse sample was reapplied to the same surface of the leaf a second time in order to rinse the same portion of the leaf surface. The aqueous sample then was extracted with equal volumes of acidified EtAc.

Acyl-HSL from the phyllosphere samples was detected using bioluminescence reporter plasmids pSB401 or pSB1075 in *E. coli* strain JM109. Volumes of EtAc extractions in 1.5-ml Eppendorf tubes were evaporated in a Savant Speed Vac. An overnight culture of JM109 (pSB401) or JM109 (pSB1075) was grown in LB with appropriate antibiotic and diluted 1:10 in LB, and 200 μ l of the diluted cell suspension was added to the round-bottom tubes (12 by 50 mm) containing phyllosphere samples. Tubes were incubated at 30°C with shaking for 5 to 6 h before bioluminescence was measured using a Turner Designs TD 20/20 luminometer. The TD 20/20 luminometer was adjusted to different sensitivities due to the varying response of the JM109 series of biosensors to their cognate AHL signal (Winson et al. 1998). Unless noted, RLU measurements were made at 30.0, 39.9, and 50.1% sensitivity for LuxR-, AhvR- and LasR-based biosensors. Luminescence is measured and given in RLU per triplicate sample. RLU were determined with a 20-s integration period. Mean values of the specific luminescence units were obtained with three independent biological samples; standard deviations, in some cases, are shown.

Growth medium samples. Selfed first- and second-generation seed from transgenic regenerates were germinated and grown in the presence of kanamycin for 21 days under standard conditions (Savka and Farrand 1992). Antibiotic-resistant progeny were transferred to the surface of cheesecloth submerged into 10 ml of Murashige and Skoog medium (MSM) in 25-by-100-mm tubes. The cheesecloth was submerged into the medium to support each plant. Samples of the MSM were collected after various days of plant growth in which the roots of the growing plants had been submerged. The collected MSM was extracted with equal volumes of acidified EtAc and, in some cases, concentrated in a Savant Speed Vac. Detection of acyl-HSL in growth medium supernatant extractions was determined using *E. coli*-based reporters in bioluminescence assays (Winson et al. 1998) and *A. tumefaciens*-based sensor NTL4 (pZLR4) in well-diffusion assays (Ravn et al. 2001), both as described in the Results section.

Soil samples. Progeny seed of transgenic genotypes were germinated and grown on MSM containing kanamycin. Seed of wild-type plants also were germinated and grown on MSM without kanamycin. After 3 weeks of growth, transgenic plants that developed a vigorous root and shoot system and wild-type plants were transferred to the surface of sterile potting soil (Miracle Grow) contained in Magenta GA-7 boxes, and these were incubated at 25°C in a growth chamber (16 h of light, 8 h of dark). Each Magenta box contained, on average, 26.3 g of commercial potting soil saturated with 40 ml of sterile, purified water before autoclaving (Savka and Farrand 1997).

After a total of 9 weeks of plant growth, plants with soil adhering to their roots were lifted gently from the Magenta boxes. The average plant crown weight was 494 $\pm$ 21.9 mg (standard error) at the time of harvest. Three types of soil samples were harvested from Magenta boxes: (i) tightly adhered soil was defined as soil adhering to roots, (ii) loosely adhered

soil was from areas between plant roots, and (iii) plant-free soil was collected from Magenta boxes in which no plants were grown. To harvest soil samples, the soil around each plant in the Magenta box was loosened by lifting soil around each plant with a sterile spatula. Tightly and loosely adhering soils were sampled. Plants then were lifted carefully and transferred to a sterile petri plate. The plant was shaken gently to remove loosely adhering soil samples. The tightly adhering soil samples then were loosened gently with a spatula. This sample represented the tightly adhering soil sample. All soil samples were extracted with four volumes of acidified EtAc, dried, and resuspended in 20- or 10- μ l volumes of EtAc. Detection of acyl-HSL in soil extractions was determined using an *A. tumefaciens* NTL4 (pZLR4) well-diffusion assay (Ravn et al. 2001).

Statistical analyses.

Data were statistically analyzed using SAS (version 9.1; SAS Institute, Inc., Cary, NC, U.S.A.).

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