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Plants genetically modified to produce *N*-acylhomoserine lactones communicate with bacteria

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***N*-acylhomoserine lactones (AHLs) play a critical role in plant/microbe interactions. The AHL, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), induces exoenzymes that degrade the plant cell wall by the pathogenic bacterium *Erwinia carotovora.* Conversely, the antifungal activity of the biocontrol bacterium *Pseudomonas aureofaciens* 30–84 is due (at least in part) to phenazine antibiotics whose synthesis is regulated by *N*-hexanoylhomoserine lactone (HHL). Targeting the product of an AHL synthase gene (*yenI*) from *Yersinia enterocolitica* to the chloroplasts of transgenic tobacco plants caused the synthesis in plants of the cognate AHL signaling molecules (OHHL and HHL). The AHLs produced by the transgenic plants were sufficient to induce target gene expression in several recombinant bacterial AHL biosensors and to restore biocontrol activity to an HHL-deficient *P. aureofaciens* strain. In addition, pathogenicity was restored to an *E. carotovora* strain rendered avirulent as a consequence of a mutation in the OHHL synthase gene, *carI*. The ability to generate bacterial quorum-sensing signaling molecules in the plant offers novel opportunities for disease control and for manipulating plant/microbe interactions.**

Keywords: *N*-acylhomoserine lactones, plant/microbe interactions, biocontrol, rhizosphere

The ability to respond to environmental cues such as nutrient availability, temperature, and pH is critical to microbial survival. Recently, it has become apparent that bacterial cells can also sense their local population density through a cell–cell communication process mediated by small diffusible molecules. This phenomenon is known as "quorum sensing" because initiation of the response depends on the population reaching a minimal "quorum" unit[1–3]. Quorum sensing is thus an example of multicellular behavior in prokaryotes and regulates diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer, and the production of virulence determinants in animal, fish, and plant pathogens[1–3]. In Gram-negative bacteria, the best understood family of signal molecules are the *N*-acylhomoserine lactones (AHLs), which vary by presence or absence of an acyl chain C3 substituent (oxo- or hydroxy-) and by length of the *N*-acyl side chain. In general, the signal generator proteins responsible for the synthesis of AHLs belong to the LuxI family, the archetypal member of which was originally identified within the *lux* operon of *Vibrio fischeri* as the gene product responsible for the synthesis of *N*-(3-oxohexanoyl)homoserine lactone (OHHL). Gene expression mediated by AHLs depends on the interaction between the signal molecule and a response regulator protein usually of the LuxR family.

In the plant pathogen *Erwinia carotovora,* OHHL synthesized by the LuxI homolog CarI (ref. 4) is responsible for regulating the production of secreted exoenzymes that degrade the plant cell wall, and the antibiotic carbapen-3-em carboxylic acid[5–7]. Similarly, the closely related AHL, *N*-hexanoylhomoserine lactone (HHL), synthesized via the *cviI* gene product of *Chromobacterium violaceum,* is responsible for the regulation of the purple pigment violacein and antifungal chitinases[8,9]. Inactivation or deletion of *luxI*, *carI* or *cviI* results in the loss of cell density–dependent bioluminescence, antibiotic production, exoenzyme synthesis or violacein production in the parent organism. Expression of the relevant operons in these mutants can be restored by the provision of an exogenous supply of AHL. Similarly, *Pseudomonas aureofaciens* 30–84 (a soil-borne bacterium that colonizes the wheat rhizosphere) inhibits the fungus *Gaeumannomyces graminis* var tritici, the causative agent of take-all disease of wheat[10]. *Pseudomonas aureofaciens* 30–84 synthesizes three phenazine antibiotics that are responsible (at least in part) for this antifungal activity. Expression of the phenazine biosynthetic operon is controlled by HHL, which is synthesized by the product of the *phzI* gene[10]. Disruption of *phzI* abolishes biocontrol activity, but this can be restored in situ by co-inoculation with an HHL-producing strain that supplies the signaling molecule in trans[11]. By synthesizing AHLs in plants, we predicted that it might be possible to manipulate the behavior of plant-associated bacteria.

Results and discussion

First, we tested the feasibility of synthesizing bacterial AHL signaling molecules in plants. Plasmid vectors were designed to express the *yenI* gene of *Yersinia enterocolitica* in either in the cytoplasm or in chloroplasts. YenI directs the synthesis of HHL and OHHL in a 1:1 ratio[12], providing the cognate AHLs for both *P. aureofaciens* and *E. carotovora*, respectively. The rationale for producing YenI in chloroplasts was twofold: First, their evolutionary relatedness to prokaryotes might more closely resemble the environment in which YenI is normally active, and second, the chloroplast might

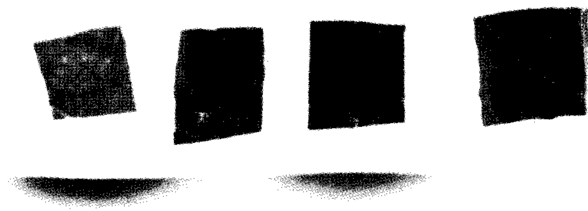

Figure 1. Production of signaling molecules by transgenic plants. Tobacco leaf segments from plants containing the *BDHERBYI* transgene were tested for the ability to synthesize AHLs. Two of the leaf segments shown tested positive as indicated by the ability of a diffusible product to induce the production of the purple pigment violacein in the *Chromobacterium violaceum* AHL biosensor strain CV026.

OHHL HHL BDHERBYI Transgenic control

Figure 2. Thin-layer chromatography analysis of AHLs synthesized by transgenic plants.

contain the necessary precursors required for AHL synthesis by YenI. These are likely to include *S*-adenosylmethionine as the source of the homoserine lactone, and either the appropriately charged acyl-acyl carrier protein or coenzyme A derivative as a source of the N-linked AHL side chain[13–15].

Two thirds of the tobacco plants transformed with the construct directing YenI to the chloroplast tested positive for AHL production as judged by their ability to induce violacein production in the AHL biosensor CV026 (Fig. 1). None of the plants in which the *yenI* construct lacked the chloroplast-targeting signal gave a positive reaction in the assay. When organic extracts of the transgenic AHL-producing plants were subjected to thin-layer chromatography (TLC), two different AHL molecules with R_f values identical to those of synthetic OHHL and HHL standards were observed (Fig. 2). The identity of both AHLs was confirmed using high-performance liquid chromatography–mass spectrometry (HPLC-MS), where the expected retention times for OHHL and HHL (9 and 13.5 min, respectively) matched the molecular ion (M + H) masses of 214 and 200, respectively (data not shown). This is consistent with the activity of YenI in homologous (*Y. enterocolitica)* and heterologous genetic backgrounds[8,12]. To estimate the levels of AHLs produced in the transgenic plants, the total molecular ion (M + H) content for each compound was determined from the HPLC-purified fractions with reference to a calibration curve constructed using synthetic OHHL and HHL standards. Measurements of 0.41 and 0.35 μg/g fresh weight were obtained for OHHL and HHL, respectively. However, the values varied between different transgenic lines according to transgene insertion sites and copy number, and within different plant organs. In crude leaf disc assays, larger zones of CV026 induction were often seen in association with older, senescent tissues. Normally, the CaMV 35S promoter is most active in younger tissue, so this may indicate that the AHLs are not readily broken down within the plant but continue to accumulate for as long as the transgene is being expressed.

Next, the ability of plant-generated AHLs to induce changes in neighboring bacteria was evaluated. The ability of the AHLs to diffuse out of intact leaves was demonstrated by placing a transgenic tobacco leaf on agar and subsequently removing it before overlaying with *C. violaceum* CV026 (Fig. 3A). The outline of the whole leaf can be seen, demonstrating that the AHLs diffused out of the entire leaf, not just the cut stem. AHLs were present not only in green tissues containing chloroplasts but also in organs such as roots, containing undifferentiated plastids. The presence of AHLs in the vicinity of roots of these plants was unmistakeable, as they were able to induce bioluminescence in a recombinant *E. coli* strain carrying an AHL-inducible *lux* operon[16] (Fig. 3B). This indicates either that root plastids are competent to synthesize AHLs, or that the AHLs synthesized in green tissues are transported to the root system. In either case, AHLs diffusing from the roots are clearly capable of signaling to nearby bacteria. Moreover, AHL-producing plant tissues are capable of restoring *G. graminis* growth-inhibiting activity to the "disarmed" *P. aureofaciens* 30–84 *phzI*⁻ strain (Fig. 3C). Finally, *E. carotovora carI (expI)* mutants, which have greatly reduced virulence on their natural host plants[6,17] were shown to infect transgenic tobacco producing AHLs (Fig. 3D).

Normally, these AHL-deficient mutants are avirulent in the tobacco system because they cannot produce the plant cell wall–degrading enzymes pectin lyase, pectate lyase, polygalacturonase, cellulase and protease[6,17]. The regulated expression of enzymes that degrade the plant cell wall only at high cell density in wild-type bacteria may contribute to the success of *Erwinia* as a plant pathogen[2]. Under aerobic conditions, *E. carotovora* infection occurs only when the microorganism has reached a critical population density. At this stage, disease progression will depend on bacterial multiplication successfully competing with the plant host defenses[18]. Thus, the production of macerating enzymes at low cell densities would not give rise to a successful infection but would result in the premature induction of the local and systemic plant defense response, which in turn would hamper subsequent infections. Such resistance to *E. carotovora* infection is seen when the plant defense response is artificially induced by the application of salicylic acid[19]. Thus, if the infecting pathogen were to encounter AHL levels that gave a false indication of the local bacterial population size, the course of the ensuing infection might be drastically altered, with the plant being able to mount a successful defense to a weak attack. To test this hypothesis, we are currently introducing AHL synthase genes into both carrot and potato, which are the natural hosts for *E. carotovora.*

The ability to synthesize bacterial signaling molecules in plants could have other important applications. For example, it may be feasible to modify the behavior of important microorganisms in the rhizosphere through AHLs produced by transgenic plants. The rhizosphere-expressed genes of the *rhiABC* operon of the symbiotic, nitrogen-fixing bacterium *Rhizobium leguminosarum*, for example, are regulated by an AHL with a C14 acyl side group containing a hydroxylated carbon in the three position and a single, unsaturated carbon-carbon bond[20,21]. Biovars of the bacterium *Pseudomonas fluorescens* are commonly used as antifungal biocontrol agents[22,23], and

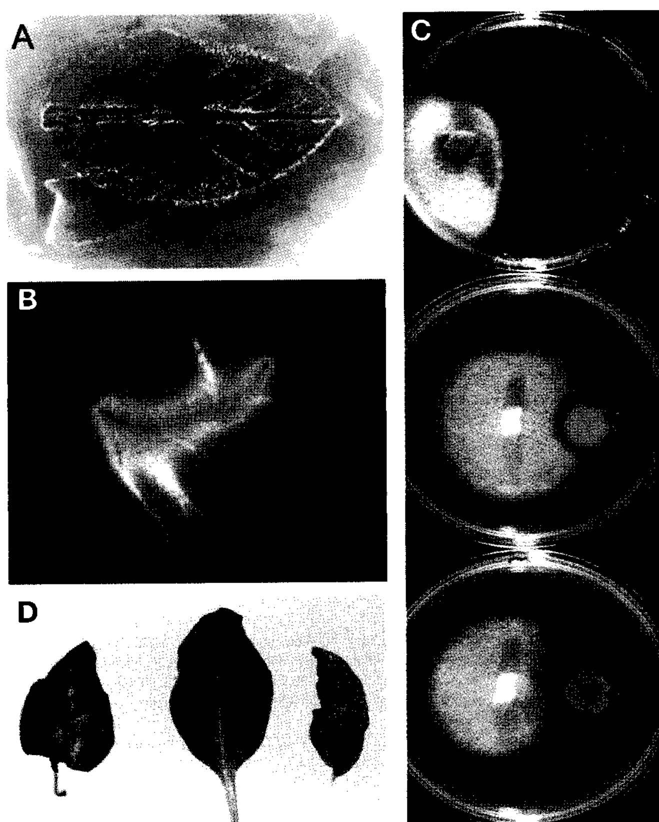

Figure 3. Bacterial responses to leaves and roots of transgenic plants. (A) A transgenic tobacco leaf was placed on an agar plate overnight, the leaf was then removed, and *C. violaceum* CV026 spread over the plate. Violacein production by the bacterium can be seen where the AHLs have diffused out of the leaf and into the agar. (B) The root of a transgenic tobacco plant grown in tissue culture under aseptic conditions was placed on an LB agar plate and overlaid with agar containing *E. coli* pSB401 (ref. 16). This AHL biosensor emits light when supplied with exogenous AHLs. (C) Partial restoration of biocontrol activity to *P. aureofaciens* 30–84I (*phzI⁻*). Leaf material from a transgenic leaf (bottom plate) and a nontransformed control (middle) were placed in wells adjacent to the bacterial colony, and *G. graminis* var tritici was inoculated opposite. The strong biocontrol activity of the wild-type *P. aureofaciens* 30–84 strain is also shown (top plate). (D) Restoration of virulence to the avirulent AHL-negative *E. carotovora* mutant PNP22. The photograph shows the leaves four days after inoculation. (1) Wild-type tobacco inoculated with wild-type *E. carotovora*. (2) Wild-type tobacco inoculated with AHL-negative *E. carotovora* mutant PNP22; (3) AHL-producing tobacco line inoculated with *E. carotovora* PNP22.

one isolate has been shown to produce at least five AHLs[24]. It might be that antifungal antibiotic production by *P. fluorescens* will prove to be quorum-sensing dependent, as it is for *P. aureofaciens*[10]. In this case, transgenic plants producing the appropriate signaling molecules might enhance the establishment of an antifungal environment or allow the use of "disarmed" strains for crop protection. Conversely, some long-chain AHLs, such as *N*-(3-oxo)-dodecanoyl-L-homoserine lactone, appear to act as competitive inhibitors of short-chain AHL perception[8]; thus, it might also be possible to inhibit the expression of certain bacterial virulence genes by the synthesis in plants of blocking molecules.

Experimental protocol

Plasmid construction. Plasmid pBDHEYI was constructed by fusing the alpha mosaic virus translation enhancer sequence (AMV) from pBI526 (ref. 25) to the *yenI* coding sequence. The *yenI* sequence had previously been amplified by PCR to create a *NcoI* site overlapping the translation initiation sequence. This changed the second amino acid from leucine to valine but did not affect the ability of the encoded enzyme to synthesize AHLs. The AMV–*yenI* fusion was cloned as a *BglII*/*BamHI* fragment into the *BamHI* site of pDH51 (ref. 26) to give pDHEYI. An *EcoRI* fragment from pDHEYI was cloned into the *EcoRI* site of the binary plant transformation vector pBIN19 (ref. 27) to give pBDHEYI. Plasmid pBDHERBYI was constructed by fusing the petunia SSU611 ribulose bisphosphate carboxylase small subunit (*rbcS*) chloroplast targeting sequence[28] to the AMV translation enhancer sequence of pBI526. A *NcoI* site was engineered to overlap the initiating ATG codon of *rbcS*. A *SphI* site was engineered to overlap the initiating ATG of *yenI* and the *yenI* coding sequence cloned into the *SphI* site of the SSU611 fragment. This site spans the cleavage site of the encoded chloroplast transit peptide. The AMV/*rbcS*/*yenI* fusion was cloned on a *BglII*/*BamHI* fragment into the *BamHI* site of pDH51 to give pDHRBYI. An *EcoRI* fragment from pDHERBYI was cloned into the *EcoRI* site of pBIN19 to give pBDHERBYI.

Generation of transgenic plants. The binary plant transformation vectors pBDHEYI and pBDHERBYI were transferred to *Agrobacterium tumefaciens* LBA4404. This was then used to transform tobacco leaf discs according to standard protocols[29]. The transgenic status of the resulting kanamycin-positive explants was confirmed by Southern blot analysis (data not shown).

Extraction and analysis of AHLs. For TLC analysis, transgenic plant extracts were made by grinding plant tissue (2 g) to a fine powder in liquid nitrogen and mixing the frozen powder with 200 ml of warm (40°C) distilled water. After 5 min, solid matter was removed by passing through filter paper and the filtrate extracted with an equal volume of ethyl acetate. The ethyl acetate layer was then dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was taken up in 500 μl of acetonitrile, and 20 μl of this was applied to a C_{18} reverse-phase TLC plate (Merck, Germany). A similar extract from an untransformed plant was also spotted onto the plate. Then, HHL (1×10^{-8} g) and OHHL (5×10^{-7} g) were applied as standards, and the chromatogram was developed with methanol/water (60:40, vol/vol) as running solvent[8,24]. After drying, AHLs were located on the TLC plate by overlaying *C. violaceum* strain CV026 in top agar as described by McClean and colleagues[8]. After 16 h growth at 28°C the presence of AHLs was indicated by localized violacein production.

For LC-MS analysis, transgenic plant extracts were made by grinding plant tissue in ethyl acetate. The supernatant was taken, the plant residue was re-extracted with ethyl acetate, the supernatants were pooled, and the process was repeated until the plant residue was white/brown in color and free of chlorophyll. The ethyl acetate layer was separated from a small plant-derived aqueous layer and dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was resuspended in 500 μl of methanol; this was brought to 60% (vol/vol) methanol with sterile distilled water and placed at -20°C overnight to precipitate out the majority of the chlorophyll. After pelleting any solid matter by centrifugation in a benchtop microfuge, the AHL-containing supernatant was partitioned against 10 volumes of ethyl acetate, and the organic phase evaporated to dryness. The residue was then taken up in 500 μl of acetonitrile. For both LC-MS and preparative HPLC, AHLs were eluted from a C_8 reverse-phase HPLC column at a flow rate of 2 ml min^{-1} using a linear gradient of acetonitrile in water (20–100%, vol/vol) over 32 min as described[30]. Samples eluting from the HPLC column were ionized by positive-ion electrospray mass spectrometry, and the spectra obtained were compared with those of the synthetic material subjected to the same LC-MS conditions.

Assay for restoration of biocontrol activity to *P. aureofaciens* 30–84I (*phzI⁻*). Leaf material from transgenic leaf and nontransformed control plants were placed in wells cut in a potato dextrose agar plate (Oxoid, Hampshire, UK), *P. aureofaciens* 30–84I (*phzI⁻*) was inoculated adjacent to the well and the plates incubated for 24 h at 22°C. The *G. graminis* var tritici was then inoculated on the opposite side of the plate and the whole incubated at 22°C for a further four days.

Test inoculations with an avirulent AHL-negative *E. carotovora* mutant. Untransformed control and transgenic BDHERBYI tobacco leaves were inoculated with the avirulent AHL-negative *E. carotovora* mutant PNP22 (refs. 5 and 6). The bacteria were applied at a high culture density ($OD_{600} = 2.5$) in a volume of 10 μl to a small wound site made with a hypodermic needle.

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