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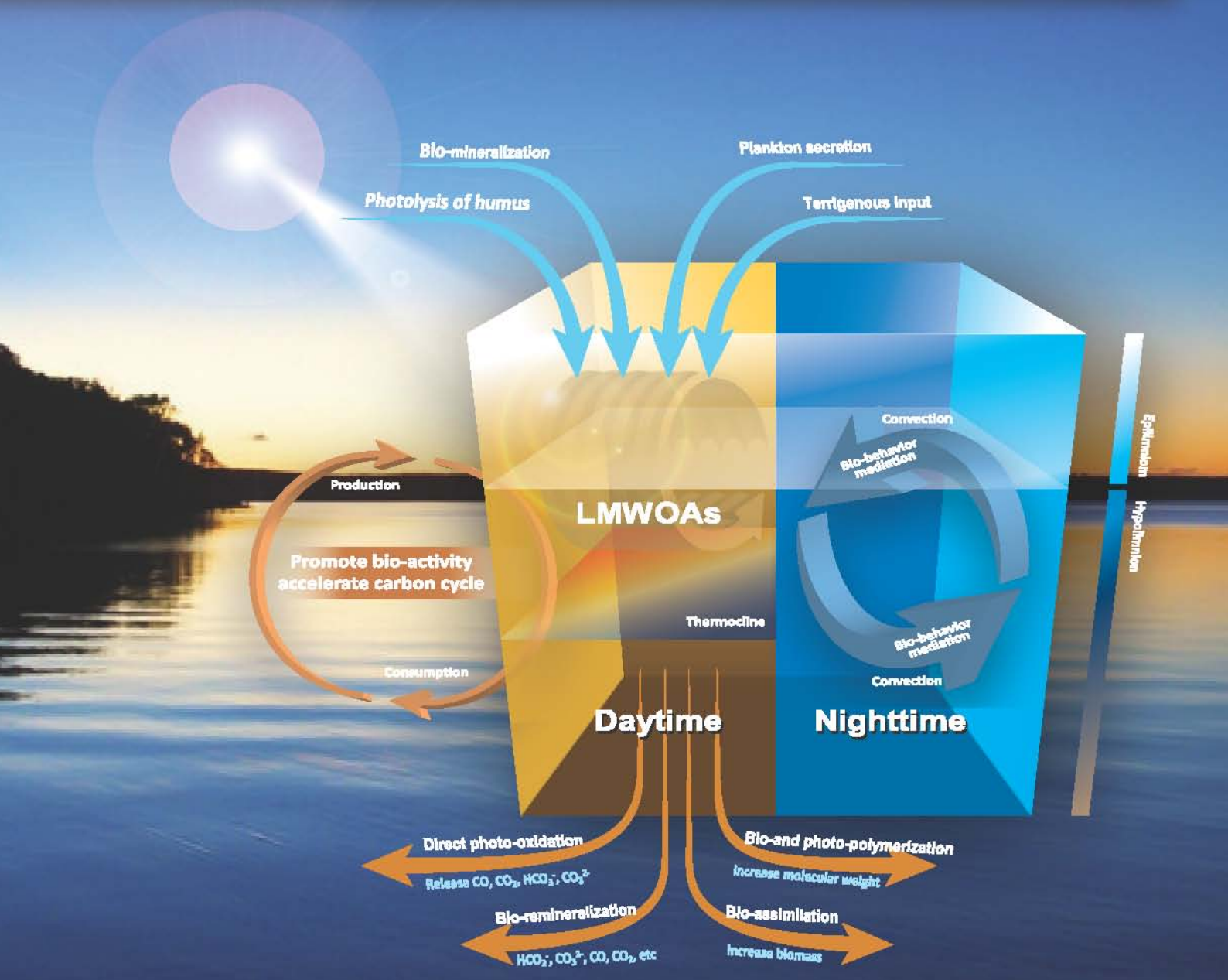

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Profile of the culturable microbiome capable of producing acyl-homoserine lactone in the tobacco phyllosphere

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Abstract

Bacterial populations coexisting in the phyllosphere niche have important effects on plant health. Quorum sensing (QS) allows bacteria to communicate via diffusible signal molecules, but QS-dependent behaviors in phyllosphere bacterial populations are poorly understood. We investigate the dense and diverse *N*-acyl-homoserine lactone (AHL)-producing phyllosphere bacteria living on tobacco leaf surfaces via a culture-dependent method and 16S rRNA gene sequencing. Our results indicated that approximately 7.9%–11.7% of the culturable leaf-associated bacteria have the ability to produce AHL based on the assays using whole-cell biosensors. Sequencing of the 16S rRNA gene assigned the AHL-producing strains to two phylogenetic groups, with Gammaproteobacteria (93%) as the predominant group, followed by Alphaproteobacteria. All of the AHL-producing Alphaproteobacteria were affiliated with the genus *Rhizobium*, whereas the AHL-producing bacteria belonging to the Gammaproteobacteria mainly fell within the genera *Pseudomonas, Acinetobacter, Citrobacter, Enterobacter, Pantoea* and *Serratia*. The bioassays of supernatant extracts revealed that a portion of the strains have a remarkable AHL profile for AHL induction activity using the two different biosensors, and one compound in the active extract of a representative isolate, NTL223, corresponded to 3-oxo-hexanoyl-homoserine lactone. A large population size and diversity of bacteria capable of AHL-driven QS were found to cohabit on leaves, implying that cross-communication based AHL-type QS may be common in the phyllosphere. Furthermore, this study provides a general snapshot of a potential valuable application of AHL-producing bacteria inhabiting leaves for their presumable ecological roles in the phyllosphere.

Key words: phyllosphere; bacteria; *N*-acyl-homoserine lactone; interaction
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Introduction

The phyllosphere, an important microbial habitat, is known to support complex microbial populations. In this environment, bacteria represent numerically dominant colonizers with populations of approximately 10^6–10^7 cells/cm^2 per leaf (Lindow and Brandl, 2003). Given that the total global terrestrial leaf surface is 10^8 km^2 (Redford et al., 2010), the ecological function of these bacteria in plant-microbe interactions is clearly important. Indeed, phyllosphere bacteria can negatively or positively affect plant health by serving as phytopathogens or biocontrol bacteria (Kishore et al., 2005a; Redford et al., 2010). Moreover, phyllosphere bacteria can promote plant health and may be important in the phyllosphere for their capacity to fix atmospheric nitrogen (Freiberg, 1998; Furnkranz et al., 2008; Kishore et al., 2005b). More generally, bacteria are unevenly distributed on leaf surfaces according to the limited and highly heterogeneous availability of nutrients (i.e., simple sugars) (Dulla et al., 2005; Hunter et al., 2010). A high density of epiphytic bacteria colonizes the limited nutrient-rich attachment sites on leaves, and microbe-microbe interactions in the phyllosphere likely occur. However, the knowledge of the interactions between the complex bacterial populations (i.e., pathogenic, neutral and beneficial bacteria) inhabiting the phyllosphere remains unclear. Understanding the underlying mechanisms of microbial communication could help reveal the productive microbe-microbe interactions and thus their influences on plant health.

The form of microbial chemical communication known as quorum sensing (QS) occurs via specific diffusible signal molecules, partly composed of *N*-acyl-homoserine

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lactones (AHLs) (Waters and Bassler, 2005), and AHL-type QS is extensively studied within members of Gram-negative bacteria (Manefield and Whiteley, 2007). Although AHL-production among rhizobacteria and phytopathogens has been intensively reported (Loh et al., 2002), the information on AHL-producing bacteria in the phyllosphere is far less documented. Recently, it has been confirmed that leaf-associated pathogenic bacteria, such as *Pseudomonas syringae*, can exhibit QS-dependent behavior, and the regulation mechanisms have been well reported in the literature (Dulla and Lindow, 2009; Quinones et al., 2005). In addition to phytopathogens, other bacterial populations, such as non-pathogenic and beneficial bacteria, can antagonize or compete with different species of bacteria in the phyllosphere and affect plant development; however, AHL signaling in this group of bacteria has been little documented and deserves research attention. Indeed, the focus on deciphering individual mechanisms has obscured the understanding of the interactions between the complex leaf-inhabiting bacterial populations from an ecological perspective. There is actually surprisingly little known about the diversity and abundance of AHL-producing phyllosphere bacterial populations, particularly with regard to the knowledge of the ecological roles of the interactions between them.

Nevertheless, a systematic understanding of leaf-dwelling AHL-active bacteria should help to highlight the importance of the interactions within phyllosphere communities that are mediated by AHL. Additionally, this knowledge will broaden biotechnological research in the field of the biocontrol of plant diseases based on AHL-producing phyllosphere bacterial resources. Therefore, the present study focused on surveying the ability of the bacteria inhabiting leaves to communicate and on profiling their phylogenetic relationships systemically. Leaf-associated strains were collected from the tobacco phyllosphere using a culture-based method, and the potential AHL-producing capacity was identified using bioassays and liquid chromatography-mass spectrometry (LC-MS). An in-depth analysis based on the 16S rRNA gene was then performed to gain insight into the phylogenetic profiles of AHL-active phyllosphere bacteria.

1 Materials and methods

1.1 Isolation of leaf-associated culturable bacteria

Leaf samples were collected from tobacco plants (*Nicotiana tabacum* L.) grown in a greenhouse. To avoid contamination, the leaves were clipped carefully from the stem directly into sterile plastic bags using sterile hand shears. Immediately after collection, the samples were transported on ice to the laboratory, and the bacteria colonizing the surface of the leaves were quickly extracted. A sample of mixed leaves (10 g) was placed into a sterile Erlenmeyer flask with 30 mL of 0.1 mol/L potassium phosphate buffer (pH 7.0) using axenic procedures and sonicated for 10 min in an ultrasonic cleaner. The mixture of the tobacco phyllosphere bacteria was then gently vortexed for 15 min on a flask shaker. The resulting suspension was centrifuged at 4°C for 10 min at 800 $\times g$ to sediment the leaf debris. Serial dilutions of the bacteria containing supernatant were spread onto culture agar plates (1% tryptone, 0.5% yeast extract and 5% sterile leaf juice) supplemented with cycloheximide (0.1 g/L) to suppress fungal growth (Madhaiyan et al., 2009). The population densities were estimated after 2 days of incubation at 30°C. The colonies were picked from the cultivated plate and purified by repeated streaking. The isolate collections obtained from the phyllosphere of tobacco were stored at –80°C in Luria-Bertani (LB) liquid broth with 30% glycerol until further processing.

1.2 Bacterial whole cell sensors for AHL

Two different sensor strains, with different optimal sensitivities to series of AHLs, were used for the bioassay. The sensor strain *Chromobacterium violaceum* CV026 produces the purple pigment violacein in the presence of short- to medium-chain AHLs (McLean et al., 1997). The bioreporter system *Agrobacterium tumefaciens* A136 expresses a *lacZ* fusion in response to a wide range of AHLs and typically produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), reflecting by β-galactosidase activity (McClean et al., 1997). The AHL over-producers *A. tumefaciens* KYC6 and *C. violaceum* ATCC 31532 were used as the positive controls in the bioassay (McLean et al., 1997). The *C. violaceum* CV026 and 31532 strains were grown in LB broth at 30°C in a rotary shaker. *A. tumefaciens* KYC6 cultivation was performed in LB broth supplemented with spectinomycin (50 μg/mL), and LB broth supplemented with spectinomycin (50 μg/mL) and tetracycline (5 μg/mL) was used as the medium for the culture of the A136 cells.

1.3 Screening for AHL-producing bacteria

Phyllosphere bacterial isolates with the ability to produce AHL were screened by a cross-streak assay against the sensor strains. The preliminary screening methods involved the cultivation of sensor strains and phyllosphere bacteria at 30°C until they grew to the exponential phase. In the bioassays, both the sensor strains and the tested isolates were streaked side by side on an LB agar plate; when A136 was used as the sensor strain, an agar plate overlaid with X-Gal (60 μg/mL) was prepared. The plates were incubated at 30°C for 2 days, and AHL-producing isolates were demonstrated by the activation of the sensors. A positive control, as described above, was performed in each bioassay experiment. Colonies on petri plates were observed to record their characteristics such as shape and colour etc. Morphology was also studied using light microscopy (phase-contrast microscope; Microscope BX51, Olympus,

Japan).

1.4 Identification of AHL in culture supernatants

A 20-mL volume of the culture of representative isolates was grown overnight at 30°C, followed by extraction with an equal volume of acidified EtAc (ethyl acetate containing 0.2% glacial acetic acid). The mixture of the culture and EtAc was then vortexed for 20 min. Following centrifugation, the combined supernatants from repeated extracts were dried under a gentle flow of filtered (0.2 μm) N_2 gas. The dried extracts were redissolved in dimethyl sulfoxide and stored at –20°C. The extracts of the bacterial cultures with AHL-like activity were examined using a cross-streak assay as mentioned above. When the AHL profile of the extracts was tested by LC-MS analysis, a Waters Micromass Q-Tof micro mass spectrometer system was used. The extracts were prepared as described previously except that the dried extracts were resuspended in acetonitrile. All of the samples were applied to a C18 column (ZORBAX Eclipse XDB-C18, 5 μm; 250 mm × 4.6 mm; Agilent, USA) and then eluted using an isocratic profile of acetonitrile-water (60:40, *V/V*), followed by a linear gradient from 60% to 100% acetonitrile in water (Morin et al., 2003). The effluent was ionized by electrospray ionization and detected in the positive-ion mode.

1.5 16S rRNA gene PCR amplification

DNA was extracted from the pure cultures using a bacterial genomic DNA isolation kit (Takara, China) according to the manufacturer's instructions. The DNA was eluted with 50 μL of the buffer solution supplied with the kit and visualized by electrophoresis through a 1% agarose gel. The amount of DNA was quantified using a NanoDrop 2000 fluorospectrometer (Thermo Fisher Scientific, Wilmington, USA) and stored at –20°C until use.

The purified genomic DNA was used as the template for the amplification of the bacterial 16S rRNA gene with universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1525R (5′-AAG GAG GTG WTC CAR CC-3′) (Xing et al., 2010). The PCR amplification was performed using an Eppendorf mastercycler gradient PCR machine with *Ex Taq* DNA polymerase (Takara, China). The amplification conditions were identical for all of the samples, with an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min, and a final elongation at 72°C for 10 min. The PCR products were examined by electrophoresis through a 1% agarose gel, followed by ethidium bromide staining. The amplified products with the correct size (approximately 1500 bp) were purified using the EZNA cycle pure kit (Omega Bio-Tek Inc., USA) for cloning.

1.6 Sequence analyses

The purified amplicons were cloned using the pGEM-T vector (Promega, Madison, USA) with *Escherichia coli* DH5α chemically competent cells (Tiangen, China) in accordance with the manufacturer's instructions. The positive colonies were randomly picked and transferred to new LB agar plates for culturing. Colony PCR was performed with the primers T7F and SP6R according to a standard protocol. The clones that yielded PCR products of the correct sizes, as determined by gel electrophoresis, were selected for sequencing. Briefly, inserts were amplified using the T7F primer, and the sequences were analyzed using an ABI PRISM 3730 sequencer (Applied Biosystems, USA).

The raw sequence data were assembled and trimmed using DNAStar software (Madison, WI, USA). Chimeric sequences were removed following the evaluation by the Mallard program (Ashelford et al., 2006). A rarefaction analysis, displaying the diversity of the phylotypes of the isolates, was performed using MOTHUR with the final clean sequences (Schloss et al., 2009). The 16S rRNA gene sequences were compared with the sequences in the GenBank database and EZtaxon database to obtain the nearest phylogenetic neighbors (Chun et al., 2007). Phylogenetic trees were constructed using the neighbor-joining algorithm implemented in MEGA version 5.0 (Tamura et al., 2011). The tree topologies were evaluated through a bootstrap analysis after 1000 bootstrap replicates of the neighbor-joining data.

1.7 BOX-PCR genomic DNA profiling

The BOX-PCR DNA fingerprint technology was performed to distinguish the microbes at the strain level, which is not sensitive in a phylogenetic analysis based on 16S rRNA gene sequence (Ishii and Sadowsky, 2009). PCR amplification was performed using the BOX1 primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) with the same amplification conditions as described previously (Sgroy et al., 2009). The PCR products were separated on a 1.5% agarose gel (SeaKem LE, USA) in 0.5× TAE buffer for approximately 16 hr, stained with ethidium bromide and photographed using a BioRad imager (BioRad, USA). The gel images were digitized and normalized using Quantity One software (BioRad, USA). The cluster analysis of the BOX fingerprints was performed using the unweighted pair-group method using average linkages (UPGMA) with the Minitab software (Minitab Inc., USA).

1.8 Nucleotide sequence accession numbers

The sequences for the 16S rRNA genes generated in this study have been submitted to the GenBank database and assigned accession numbers JQ779035 to JQ779066.

2 Results

2.1 Isolates with AHL-activity in the phyllosphere

To analyze the AHL-producing potential of the isolated bacteria originating from the tobacco phyllosphere,

a cross-feeding assay was tested with the sensor strains *A. tumefaciens* A136 and *C. violaceum* CV026. Representative bioassay results are shown in **Fig. 1**. In the bioassay, a total of 66 strains out of the 564 isolates displayed the ability to produce AHL, and 46 out of the 580 strains showed AHL production in the other independent assay. Overall, the initial screening of the isolates revealed that the AHL-producers represented 7.9%–11.7% of the culturable leaf-associated bacteria.

To evaluate whether the sampling size was sufficient, a rarefaction analysis was performed based on the 16S rRNA sequences of randomly selected AHL-producing strains. The rarefaction analysis resulted in a decrease in the slope and confidence interval of the curve (**Fig. 2**), revealing that the major portion of the diversity of the isolated culturable phyllosphere AHL-producing bacteria was covered. Moreover, the positive induction of the sensor strains by the extracts of the isolates was characterized (**Fig. 1**). It is important to note that the extracts of the major AHL-active isolates showed positive induction, whereas those of several strains had a weak or even undetectable AHL activity (data not shown). Only the isolate, both itself and its culture extracts having AHL activities, was chosen for further phylogenetic analyses.

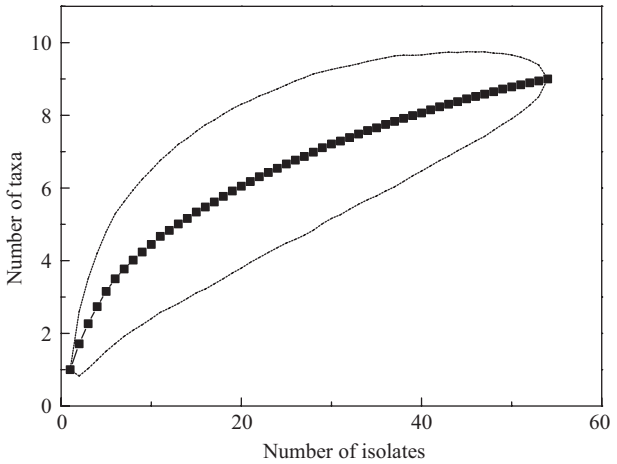

Fig. 2 Rarefaction curve of the isolate collection. The confidence interval is indicated as a dashed line.

2.2 Phylogenetic diversity of AHL-producing strains

Morphological characteristics of the isolated AHL-producing strains were given in **Table 1**. Pigmentation of the colonies was different including translucent, opaque, cloudy. Most of colonies had entire margins and few of them had lobate margins. Different isolates also has distinct cell shape and arrangement. Most isolates were rod shaped, and some were coccus shaped (**Table 1**). A phylogenetic tree of the full 16S rRNA gene sequences was constructed for the phyllosphere AHL-active strains (**Fig. 3**). The isolates were assigned to two phylogenetic groups, with Gammaproteobacteria as the predominant group, followed by Alphaproteobacteria. In the Alphaproteobacteria, only one *Rhizobium* genus with 2 positive

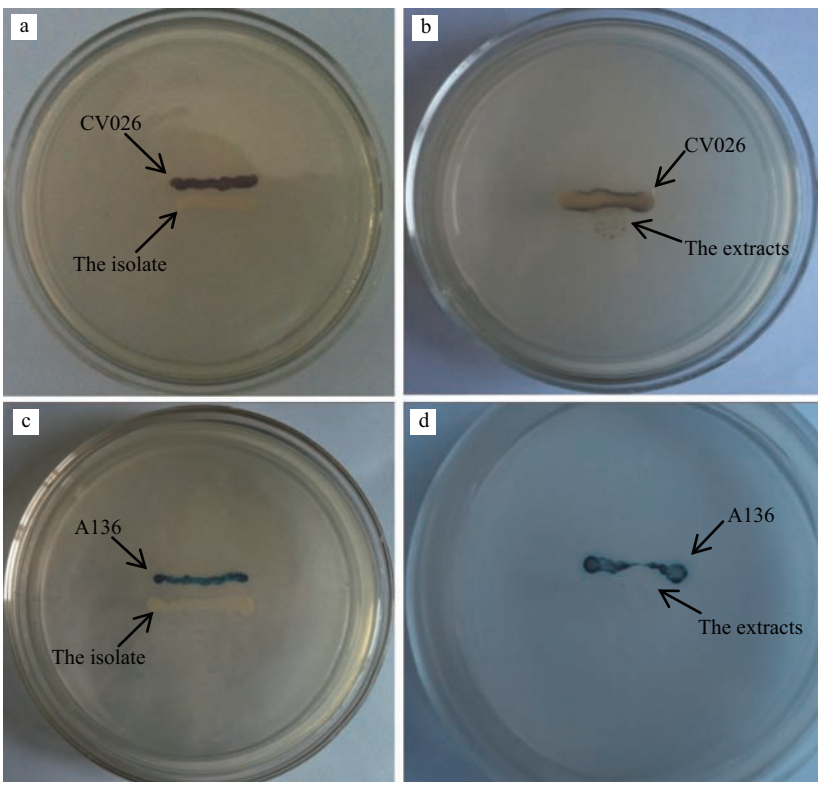

Fig. 1 Representative bioassays for AHL-activity. The isolates (a, b) and extracts of their culture (c, d) were tested using cross-feeding assays. Biosensor strains *C. violaceum* CV026 (a, c) and *A. tumefaciens* A136 (b, d).

Table 1 Characteristics of AHL-producing bacteria isolated from leaf surface of tobacco

Isolate	Form	Colour	Texture	Elevation	Margin	Cell shape	Arrangement
NTL306	Irregular	Opaque	Butyrous	Raised	Undulate	Short rod/coccus	Single
NTL465	Irregular	Opaque	Butyrous	Convex	Undulate	Rod/short rod	Single
NTL113	Circle	Translucent	Butyrous/moist	Raised	Undulate	Rod	Single
NTL233	Irregular	Translucent	Mucoid	Raised	Undulate	Short rod/coccus	Single
NTL135	Irregular	Translucent	Mucoid	Flat	Undulate	Short rod	Single/small clusters
NTL214	Circle	Translucent	Butyrous	Raised	Entire	Short rod/coccus	Single/small clusters
NTL125	Irregular	Translucent	Butyrous	Flat	Undulate	Rod	Single
NTL134	Irregular	Translucent	Butyrous	Raised	Entire	Rod	Single/pairs
NTL160	Irregular	Translucent	Butyrous	Flat	Entire	Rod	Tetrades
NTL206	Irregular	Translucent	Moist	Raised	Entire	Rod	Single
NTL208	Irregular	Translucent	Butyrous	Flat	Entire	Rod	Single
NTL240	Circle	Opaque	Butyrous	Convex	Entire	Rod	Single/pairs
NTL255	Irregular	Opaque	Butyrous	Raised	Entire	Short rod	Single/pairs
NTL264	Irregular	Translucent	Moist	Raised	Undulate	Rod	Single
NTL414	Circle	Translucent	Viscous	Flat	Entire	Rod	Single
NTL433	Circle	Translucent	Butyrous	Flat	Entire	Rod	Single
NTL501	Circle	Translucent	Butyrous	Flat	Entire	Short rod	Single
NTL126	Irregular	Translucent	Butyrous	Convex	Entire	Short rod	Pair/tetrades
NTL538	Circle	Translucent	Butyrous	Raised	Entire	Short rod	Single/pairs
NTL223	Circle	Translucent	Moist	Raised	Entire	Short rod/coccus	Pair
NTL236	Irregular	Cloudy	Moist	Raised	Lobate	Rod	Single
NTL237	Irregular	Cloudy	Moist	Raised	Filiform	Rod ellipsoid	Single/pairs
NTL246	Irregular	Translucent	Butyrous	Convex	Lobate	Short rod	Pair/tetrades
NTL247	Circle	Opaque	Butyrous	Convex	Entire	Short rod	Single/pairs
NTL249	Irregular	Translucent	Mucoid	Flat	Filiform	Short rod	Pair
NTL337	Irregular	Opaque	Butyrous	Raised	Undulate	Short rod	Pair/tetrades
NTL338	Irregular	Opaque	Butyrous	Convex	Lobate	Short rod	Pair/small clusters
NTL342	Irregular	Opaque	Butyrous	Convex	Entire	Short rod	Single/pairs
NTL344	Irregular	Translucent	Butyrous	Raised	Lobate	Short rod	Pair/small clusters
NTL235	Irregular	Translucent	Moist	Convex	Filiform	Rod	Pair
NTL215	Irregular	Translucent	Moist	Umbonate	Undulate	Short rod/coccus	Single/small clusters
NTL244	Irregular	Translucent	Butyrous	Umbonate	Lobate	Short rod	Pair

isolates was present, whereas a 93% proportion of the AHL-producing bacteria belonging to the Gammaproteobacteria; were assigned to the genera *Pseudomonas, Acinetobacter, Citrobacter, Enterobacter, Pantoea* and *Serratia*. Among these genera, *Pseudomonas* and *Serratia* were the two predominant groups, comprising 14 and 10 strains, respectively. Furthermore, 10 isolates shared 97%–100% identity based on the 16S rRNA gene sequence analysis and were closely related to the type strains *Pseudomonas brassicacearum* and *Pseudomonas thivervalensis*, grouping together in a single cluster (cluster I) (**Fig. 3**).

This group was further differentiated by BOX fingerprints at the strain level (**Fig. 4**). The BOX fingerprints generated from the genomic DNA of the isolates from cluster I in **Fig. 3** revealed 6 different BOX types on the basis of a similarity cut-off of 90%. Isolates NTL206 and NTL264 showed similar BOX pattern and clustered together. Nearly identical BOX profiles were found for strains NTL240, NTL414, NTL433 and NTL501, which were all closely related; by sequence analyses to *P. brassicacearum* (99.6%) and grouped in to a single tight major cluster, as shown in **Fig. 4**.

2.3 Characterization of AHLs in active isolates

The AHL profiles of the positive isolates were analyzed relying on rapid assays involving different AHL-responsive sensor strains. The CV026 biosensor responds to the presence of short-chain AHLs with acyl chain lengths of C4–C6. In the contrast a broad range of AHL molecules (C6–C14), particularly almost all of the moderate-chain 3-oxo-AHLs, can activate the A136 biosensor. The positive isolates with each AHL pattern detected by the biosensors are shown in **Fig. 3**. The majority of the positive strains, approximately 96%; phylogenetically disrupted among Alpha- and Gammaproteobacteria, could active the A136 biosensor. However, surprising AHL patterns of the *Serratia* sp. were observed for their ability to activate both the CV026 and A136 sensors, except for strain NTL249, which has a clearly unique AHL profile. Interestingly, strains NTL215 and NTL244, belonging to *Rhizobium radiobacter*, displayed distinct AHL profiles for their positive induction of A136 and both sensors, respectively. As the predominant group, all of the *Pseudomonas* sp. isolates could active the A136 biosensor. In this group, a representative *Pseudomonas* sp. strain NTL223, the numerically dominant positive isolate, showed a remarkable

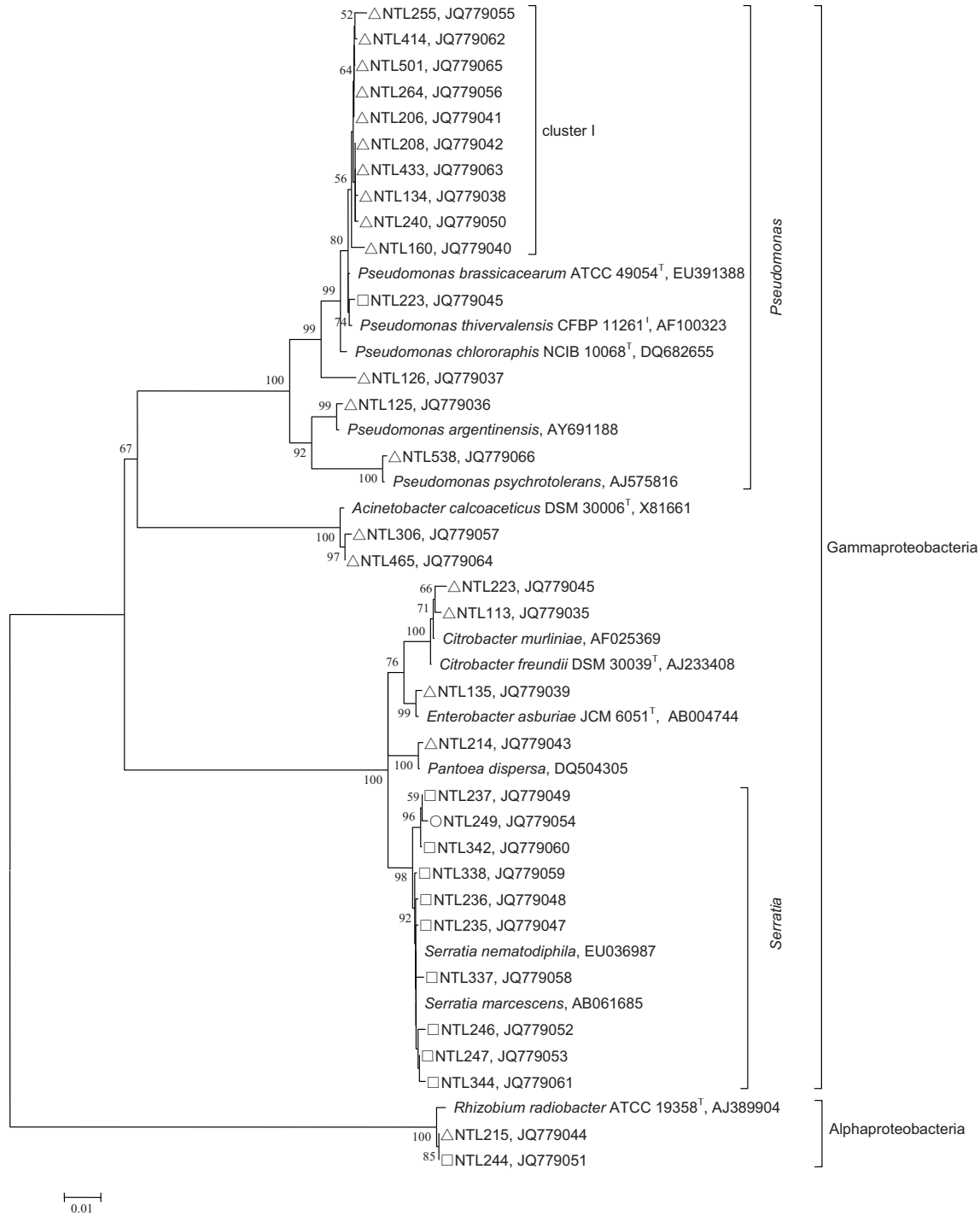

Fig. 3 Phylogenetic relationship of the AHL-producing isolates. The tree was constructed using the neighbor-joining method based on the 16S rRNA gene sequences of the isolates and reference taxa. The circles denote that the isolates can activate *C. violaceum* CV026, the triangles represent the positive induction of *A. tumefaciens* A136, and the squares represent the inductive activity detected by these two biosensors. Accession numbers are indicated after the name of the reference sequences. The values represent the relative proportions that a branch appeared in 1000 bootstrap replications. Scale bar, 0.01 relative sequence divergence.

AHL pattern for its prominent AHL activities in terms of the intensity of the pigmentation for both of the distinct sensors. The AHL profile of the active extracts from strain NTL223 was also subjected to identification by LC-MS. **Figure 5** shows the LC chromatogram of the putative AHL-active extracts of the NTL223 culture. The selected peak with a 3.15 retention time had a mass spectrum with a molecular ion of *m/z* 102, a common product ion resulting from the dissociation of AHL. Molecular ions of *m/z* 135 $[M+Na-101]^+$ and *m/z* 196 $[M+H-H_2O]^+$ were also detected (**Fig. 5**), suggesting the presence of 3-oxo-hexanoyl-homoserine lactone in the extract.

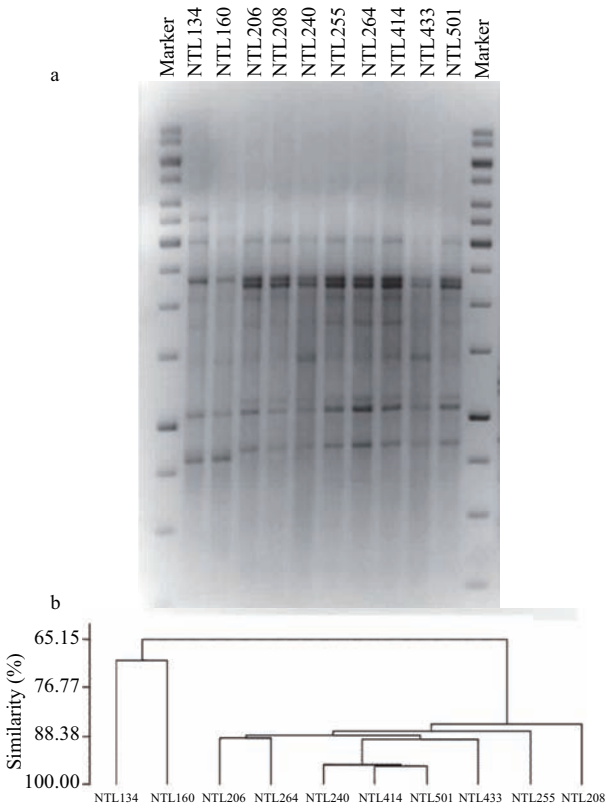

Fig. 4 Comparing the similarity of BOX-PCR genomic DNA fingerprints. (a) the result of the electrophoresis of PCR products from the isolates grouped in cluster I shown in **Fig. 3** (marker: 1 kb DNA ladder); (b) the dendrogram of clustering based on the BOX-PCR patterns derived using UPGMA.

3 Discussion

As a special microbial habitat, the phyllosphere harbors incredibly large populations of microorganisms that are predominated by bacterial colonizers with populations of approximately 10^6–10^7 cells/cm^2 per leaf. The diversity of these leaf-dwelling bacteria together on the leaf surfaces can have important influences on plant health (Redford et al., 2010). Although solid data to support the hypothesis for the competition and collaboration of epiphytic bacteria occurring *in situ* are lacking, the resident bacterial populations on the surfaces of leaves likely have the ability to prevent the colonization of exogenous microbes (Hunter et al., 2010). Indeed, bacteria can communicate with each other and often utilize AHL-type QS (Uroz et al., 2009), highlighting this possibility. *N*-acyl homoserine lactones secreted by rhizobia can also stimulate leguminous plants to develop nodules (Jones et al., 2008). Considering the potential ecological function of QS in the niche microenvironments that occur in the phyllosphere, we focused on the isolation of leaf-associated bacteria and elucidated their ability to communicate.

The culturable bacteria isolated from tobacco leaves included AHL-producing bacteria with a proportion of 7.9%–11.9%, indicating that leaf-associated bacteria with the ability to synthesize AHLs might be common in the phyllosphere. Indeed, the proportion of bacterial phyllosphere AHL-producers is likely to be even higher, as the rarefaction analysis indicated that only the major part of those strains was covered in this survey (**Fig. 2**). It is not surprising that a high relative abundance of AHL-active bacteria inhabit the leaves, perhaps because of their survival strategies in the stressful phyllosphere environment due to the fluctuations in the physical conditions. Some data exist in support of this claim, e.g., an even smaller population size of *P. syringae* on dry leaves could accumulate sufficient AHL signal molecules to activate the QS involved in epiphytic fitness, in the sense that it contributes to the survival on leaves often harboring limited surface water (Dulla and Lindow, 2008). Similarly, a highly heterogeneous availability or the scarcity of surface-deposited nutrients on leaves, such as sugars, also constrains bacterial colonization and success in the phyllosphere (Lindow and Brandl, 2003; van der Wal and Leveau, 2011). As proposed by Dulla et al. (2005), *P. syringae* AHL-based QS that regulates motility could favor their own survival in this phyllosphere nutrient desert. Clearly, the AHL-active isolates might represent the microbial populations that are easily adapted to the phyllosphere environment, even though our findings underestimate the true prevalence of bacterial AHL-producers.

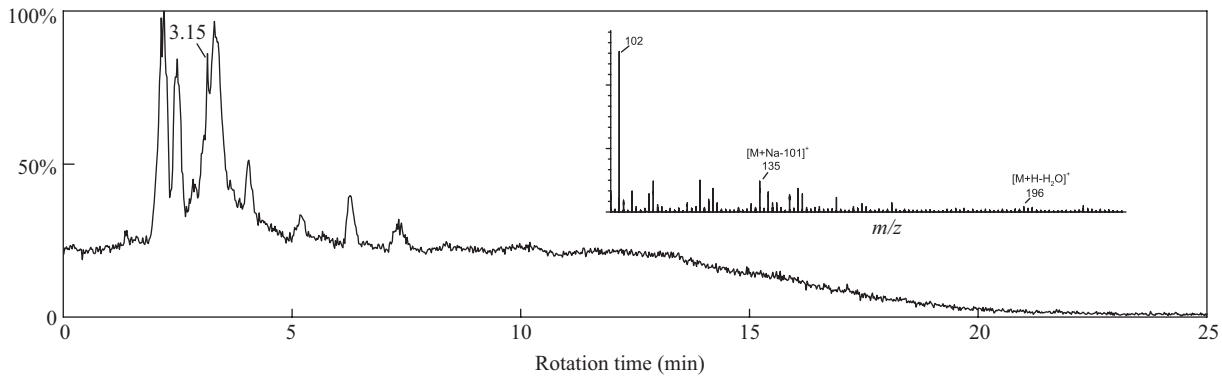

Fig. 5 LC-MS chromatogram of 3-oxo-hexanoyl-homoserine lactone occurring in strain NTL223 extracts. The peak with a retention time of 3.15 corresponds to 3OC6HSL.The inset graph denotes the fragmentation pattern of the mass spectra.

The culturable phyllosphere bacteria producing AHL included representatives primarily clustered with the Gammaproteobacteria (93% of the AHL-active population) (**Fig. 3**). Furthermore, the Pseudomonads were the predominant family detected in the present study (approximately 60%), followed by Enterobacteriaceae family members (approximately 36%). Interestingly, the phyllosphere populations from many diverse plant species seemed to be dominated by Gammaproteobacteria, phylogenetically distributing among the Pseudomonads and Enterobacteriaceae family (Kuklinsky-Sobral et al., 2004; Redford and Fierer, 2009; Whipps et al., 2008). *N*-acyl homoserine lactone producing strains were more frequently observed in the common phylogenetic group associated with the phyllosphere, though a more intimate relationship between them requires more ample evidence for support. It is important to note that the AHL-producing strains being restricted to the Alpha- and Gammaproteobacteria is consistent with the data in previous studies, as an AHL-producing bacterium outside the subphyla Alpha-, Belta- and Gammaproteobacteria has never been obtained (Manefield and Whiteley, 2007). Two alphaproteobacterial isolates, NTL215 and NTL244, closely related to *Rhizobium radiobacter*, proved to be AHL-active. Using LuxIR-type QS circuits, *R. radiobacter* can synthesize *N*-(3-oxooctanoyl)-L-homoserine lactone, as is the case in some other QS systems (Waters and Bassler, 2005). The majority of the signal-positive gammaproteobacterial strains are closely affiliated with the genera *Pseudomonas* and *Serratia*, whereas the remaining isolates clustered with *Acinetobacter, Citrobacter, Enterobacter* and *Pantoea* genera. To the best of our knowledge, leaf-associated strain NTL465 and NTL306, closely related to *Acinetobacter calcoaceticus*, appear to be new AHL producers, though a homolog of AHL synthase was recently deduced from the genome sequence of a phenol-utilized *A. calcoaceticus* (Zhan et al., 2011).

It is not surprising that the proportion of the genus *Pseudomonas* accounted for the majority of the AHL-producing members in the phyllosphere, as plant-associated *Pseudomonas* spp. are well known QS species (Elasri et al., 2001; Loh et al., 2002). Accordingly, more than 12 different plant-associated *Pseudomonas* species have the capacity to synthesize AHLs (Elasri et al., 2001). However, these *Pseudomonas* species with different traits regulated by one or more QS systems can have neutral, negative or positive effects on their host plants (Kim et al., 2011). For instance, under the control of two different QS systems (Zhang and Pierson, 2001), a *Pseudomonas aureofaciens* strain can dwell on the plant surface and effectively prevent disease in wheat (Loh et al., 2002). Despite this, the *Serratia* genus, with one known AHL-producing group, is found in a broad range of habitats, such as the soil, water and plants (Whitehead et al., 2001), yet their role in the phyllosphere environment remains unclear. Akutsu and colleagues reported that a *Serratia marcescens* strain isolated from tomato the phylloplane was very effective as a biological control agent (Akutsu et al., 1993). The fact that some of the AHL-active isolates found in the present study were phylotypes related to *S. marcescens* implied that these bacteria might have important ecological functions in the phyllosphere. In the present study, a high population size and diversity of bacteria capable of AHL-driven QS were found to cohabit in the phyllosphere, indicating that QS may play a potential role in this harsh environment.

It is noteworthy the A136 sensor strain could be induced by all of the leaf-associated AHL-positive strains, perhaps because of the sensor responding to a broader range of AHLs. However, the deeper analysis of the bioassay results revealed clear differences between the *Pseudomonas* and *Serratia* genera. The AHL-producing *Pseudomonas* isolates mainly activated the A136 sensor strain, whereas all of the AHL-producing strains belonging to *Serratia* could be assayed positively with the CV026 strains as the reporter. Based on the 16S rRNA gene sequences and BOX genomic fingerprints analyses, the bioassay pattern is similar at the strain level, as shown in **Fig. 3**. The occurrence of positive bioassays using both reporters induced by different isolates may favor their cross communication within a shared phyllosphere niche. For instance, leaf isolates with AHL-activities can cross talk with AHL-producing *P. syringae*, as recently reported (Dulla and Lindow, 2009). In agreement with previous studies, most of the pseudomonads had dominant properties of 3OC6HSL activity (Cha et al., 1998), evidenced by the numerically dominant *Pseudomonas* sp. isolate NTL223 detected by LC-MS. Interestingly, Walker et al. (2006) demonstrated that the 3OC6HSL signal molecule could increase the resistance of *Vibrio fischeri* to UV light, a result that is congruent with the opinion that the QS molecule is no more than a signal (Schertzer et al., 2009). Although it is unclear whether 3OC6HSL secreted by phyllosphere isolates is associated with UV protection on the leaf surface where the bacteria are exposed to sunlight, our study provides a general description of the diversity and the presumable ecological role of the AHL-producing bacteria inhabiting leaves. The challenge for future studies will be to elucidate the role of AHL-driven QS in the phyllosphere niche and its influence on plant health.

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