Identification and Characterization of a Second Quorum Sensing System in Agrobacterium tumefaciens A6

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Running Title: Second QS system in A. tumefaciens

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Abstract

Quorum sensing (QS) is a widespread mechanism of bacterial communication in which individual cells produce and respond to small chemical signals. In Agrobacterium tumefaciens, an acylhomoserine lactone (AHL) dependent quorum sensing mechanism is known to regulate the replication and conjugation of the tumor-inducing (Ti) plasmid. Most of the quorum-sensing regulatory proteins are encoded within Ti plasmid. Among them, TraI is the LuxI-type enzyme synthesizing the QS signal 3OC8HSL, TraR is the LuxR-type transcriptional factor perceiving 3OC8HSL, and TraM is an anti-activator that antagonizes TraR. Recently we identified a TraM homologue encoded by the traM2 gene in the chromosomal background of A. tumefaciens A6. In this paper, we further identified additional homologues (TraI2 and TraR2) of TraI and TraR in this strain. We showed that similar to TraI, TraI2 could predominantly synthesize the QS signal 3OC8HSL. We also showed that TraR2 could recognize 3OC8HSL and activate the tra box-containing promoters as efficiently as TraR. Further analysis showed that traM2, tral2 and traR2 are physically linked on a mobile genetic element which is not related to Ti plasmid. These findings indicate that A. tumefaciens A6 carries a second QS system (QS2) which may play a redundant role in regulation of the replication and conjugation of Ti plasmid.

Key words: Quorum sensing; Ti plasmid; TraI2; TraR2; conjugation
INTRODUCTION

Quorum sensing (QS) is a bacterial community genetic regulatory mechanism that controls diverse biological functions in different bacterial species. Among the various bacterial QS systems reported, the most characterized one is the acylhomoserine lactone (AHL) based QS system. In this system, the LuxI (I)- and LuxR (R)-type proteins appear to be the central components. The I-type protein is the AHL synthase and the R-type protein is the AHL-responsive transcription factor, which are conserved in different bacterial species containing AHL-based QS systems (1). In most cases, at low bacterial population density the I-type enzyme produces a basal level of AHL signals, which accumulate as bacterial cells proliferate and interact with a cognate R-type transcription factor. Subsequently, the R-AHL complexes induce higher level expression of I-type enzymes, which boosts AHL production and activate the transcriptional expression of other QS-dependent genes (2). It is through QS that individual bacterial cells could behave as a coordinated community in performing various biological activities, such as producing secondary metabolites, synthesizing virulence factors and developing biofilms.

*Agrobacterium tumefaciens* has been extensively studied as a pathogen model for investigation of microbe-host interactions. Upon perceiving the chemical signals produced by plant hosts, *A. tumefaciens* infects a variety of plants and causes the crown gall diseases, which result in substantial losses of agricultural production worldwide. During the infection, a piece of DNA fragment (T-DNA) is transferred from the bacterial cells into the plant host cells and integrated into the chromosomal DNA.
(3-5). T-DNA, together with the genes associated with its interkingdom gene transfer, is located on the Ti plasmid (2). Interestingly, many environmental *Agrobacterium* isolates do not harbor the Ti plasmid and hence are avirulent. Therefore, conjugative transfer of the Ti plasmid from the pathogenic strains to plasmid-free strains could play a key role in maintaining and expanding the population of infectious *A. tumefaciens*.

(6-8). In *A. tumefaciens*, the key QS regulators are encoded by the Ti plasmid, which includes TraI and TraR. TraI synthesizes the AHL signal, N-3-oxooctanoyl-Lhomoserine lactone (3OC8HSL), which binds to and activates TraR (9-11). TraR then binds to the palindromic *tra* box and thereby activates a number of operons that encode proteins necessary for Ti plasmid replication and conjugation (7, 12-14). In this context, the QS system of *A. tumefaciens* is similar to the prototype LuxI-LuxR QS system of *Vibrio* species.

However, the regulatory mechanisms of the *A. tumefaciens* QS system appear more complicated than the prototype mechanism. Firstly, this QS system is normally not active until the bacterial cells detect the conjugative opines produced by the crown gall tumors incited by the pathogen (15). Furthermore, when the TraR level is low, TraM binds to TraR and forms an inactive complex which sets off the QS system until the cell density is high (16-18). TraM, functioning as a TraR antiactivator, has been identified in part of the agrobacterial species. Therefore, species-specific opines and TraM constitutes additional regulatory components for the QS system of *A. tumefaciens* and ensures the Ti plasmid conjugation occurs only under certain conditions. In addition to this species-specificity, the QS system of *A. tumefaciens* also
displays strain-specific features. For example, in octopine-type strains, the conjugative
opine for the QS-dependent Ti plasmid conjugative transfer is octopine (19); while in
nopaline-type strains, it is agrocinopines A and B (20). In addition, in nopaline strain
C58, traR is a member of a five-gene operon of pTiC58, which is expressed from a
promoter regulated by the transcriptional repressor AccR. Repression by AccR is
relieved in the presence of agrocinopine A or B and the operon, including traR, is
expressed (19). In contrast, in octopine strains, traR is located in a 14-member operon
that is regulated by the transcription factor OccR (21). OccR acts either as a repressor
or an activator depending on the absence or presence of octopine, respectively, by
binding to different positions of the promoter of the traR operon (22). Furthermore, in
octopine strains, the QS system is also negatively regulated by TrlR, a truncated
version of TraR. TrlR antagonizes the TraR activity by formation of inactive
heterodimers (23, 24). In contrast, no TrlR homolog is found in nopaline strains.
Moreover, while TraM encoded by Ti plasmid is a conserved quorum-sensing
modulator in both octopine- and nopaline-type strains of A. tumefaciens, a TraM
homologue (TraM2) has recently been identified as an extra TraR antiactivator for QS
regulation. Like TrlR, TraM2 only exists in some of the octopine strains (25). Put
together, these findings have illustrated the complexity and genetic variations of the A.
tumefaciens QS system.

In this paper, we aimed to further study the regulation of QS in A. tumefaciens.
Using genetic approaches, we showed that traR2 and tral2 encode a second QS
system in A. tumefaciens strain A6. Bioinformatic and biochemical analyses showed
that TraI2 is a functional homologue of TraI predominantly synthesizing 3OC8HSL and 
TraR2 is a functional homologue of TraR perceiving 3OC8HSL and activating the QS-
responsive genes. Conjugal transfer assay showed 
traR2 and 
traI2, together with the 
previously identified 
traM2, are located on a mobile genetic element. Phylogenetic 
analysis suggested that this second QS system may represent an ancient paradigm of 
the QS systems of 
Rhizobial species.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains, 
plasmids used in this study are listed in Table 1. A. tumefaciens strains were grown at 
28°C in LB medium (per liter contains Bacto-tryptone 10 g, yeast extract 5 g, NaCl 10 
g, pH 7.0), or in BM minimal medium (basic minimum nutrient with 0.2% mannitol 
added as sole carbon source and 0.2% ammonia sulphate as sole nitrogen source 
unless otherwise indicated) (26). For bacterial growth assay, mannitol was replaced by 
octopine (0.2 g/L). E. coli strains were grown at 37°C in LB medium. Antibiotics were 
added at the following concentrations when required: kanamycin, 50 µg/ml (A. 
tumefaciens) or 100 µg/ml (E. coli); tetracycline, 5 µg/ml (A. tumefaciens) or 10 µg/ml 
(E. coli); rifampicin, 100 µg/ml; ampicilin, 100 µg/ml; chloramphenicol, 50 µg/ml.

DNA manipulation and plasmid construction. Plasmids were purified using 
the Plasmid Miniprep Kit as recommended by the manufacturer (Qiagen). PCR 
(Polymerase Chain Reaction) product purification and DNA recovery from agarose gel 
were carried out with the QIAquick PCR Purification Kit and QIAquick Gel Extraction 
Kit (Qiagen), respectively. For gene deletion construct pK18-traI, the DNA fragments
flanking \textit{traI} were separately amplified from \textit{A. tumefaciens} strain A6 with two PCR primer pairs 5'- GCTCTAGAAGTGCGGACAGCG / 5'- CGGGATCTTGTGTTGATTTCGCG and 5'- CGGGATCCGATTACGTGACG / 5'- GCTCTAGATCTCGGCAATGATGCGATTG (enzyme cut sites underlined), digested with BamHI and ligated with each other for PCR amplification using the primer pair 5'- GCTCTAGAAGTGCGGACAGCG and 5'- GCTCTAGATCTCGGCAATGATGCGATTG. The PCR products were rescued from agarose gel and cut by XbaI for subsequent ligation with XbaI-digested vector pK18mobsacB. The resultant plasmid was screened by PCR and confirmed by DNA sequencing. For preparation of the expression constructs pLA-\textit{traI} and pLA-\textit{traI2}, \textit{traI} and \textit{traI2} were respectively amplified using the PCR primer pairs 5'- CGGGATCTTGTGTTGATTTCGCG and 5'- CGGGATCCGATTACGTGACG / 5'- GGAATTCCATGCGGACACCGTCTC and 5'- GGAATTCCATGCGGACACCGTCTC (enzyme cut sites underlined), cut by BamHI and EcoRI and then inserted in pLAR3 vector by placing \textit{traI} and \textit{traI2} separately under the control of the \textit{lac} promoter carried by the vector. For construction of the expression plasmid pDSK-\textit{traR2}, the \textit{traR2} gene was cloned from \textit{A. tumefaciens} A6 with the PCR primers 5'- ACCCACCTTACACATCAAGC / 5'-CACAAGTGCGCATGTAT (enzyme cut sites underlined and translational initiation sites italicized), enzymatically digested and linked into the vector pDSK by placing the gene under the control of the vector-borne
lac promoter. The pLA-traG::lacZ and pDSK-traR were isolated from the A. tumefaciens NT1(traR, tra::lacZ749) (9).

**Genetic manipulation of A. tumefaciens.** Plasmids were transformed into A. tumefaciens by electroporation unless otherwise specified. The in-frame deletion of tral in strain A6(traM588) were carried out following the procedures described previously (25). Conjugation analysis was performed on solid plates with octopine (0.2 g/L) induction as reported previously (25). Tn5 transposon mutagenesis of A. tumefaciens was performed as described previously (27). Ti plasmid conjugation transfer efficiency was examined with octopine induction on solid plates following the procedure as described previously (25). Tumorigenicity assay of A. tumefaciens on plants was conducted as previously described (28).

**Quantification of β-galactosidase activity.** Quantitative analysis of β-galactosidase activity in bacterial culture of A. tumefaciens strains was conducted according to the method previously described (29). The β-galactosidase activity was measured and expressed as units per $10^9$ CFU.

**Detection of AHLs.** The amount of AHL signals produced by bacterial cells was determined as described previously (30). The AHL indicator strain was A. tumefaciens NT1(traR, tra::lacZ749) unless other stated. Bacteria were grown overnight at 28°C for measurement of AHL production and the amount of AHL production was calculated and expressed as the equivalent of 3OC8HSL as described previously (31, 32). TLC analysis of AHLs was carried out as reported previously (33). Briefly, bacterial strains were grown in 90 ml BM media for overnight and the supernatants were extracted by 200 ml ethyl acetate. The organic extracts were then
condensed down to 180 µl in methanol. Then 2 µl samples were separated by 50:50 (methanol: water) on C18 TLC plate with 1µl (1 µM) standard 3OC8HSL. The signals were visualized by overlaying of NT1(traR, lacZ749) on the TLC plate.

**RNA preparation and RT-PCR analysis.** Bacterial strains were cultivated in minimal medium at 28°C with shaking at 200 rpm. When the OD₆₀₀ value reached approximately 0.5, cells were collected and total RNAs were isolated using the RNeasy Mini kit (QIAGEN). Residual DNAs presented in the RNA samples were removed by digestion with the RNase-free DNase I. Quantity and quality of the RNA samples were examined with Nanodrop® ND-1000 (Nanodrop Technologies) and by agarose gel electrophoresis. An aliquot of 0.2 µg total RNAs was serially 10-time diluted and used as template for one-step RT-PCR analysis (QIAGEN). PCR primer pairs used for RT-PCR analysis were: traM2, 5'-GCAGGGACTTCCAGCG / 5'-AAACCCAGAAGTCCAAACAGC; traR2, 5'-GACTGCACAATACCCGGCC / 5'-TCCGCTCCAGGCTAAACTC; traI2, 5'-CCACCTATGTCCTTGCG / 5'-CGGTATTGTCAATCGCTACG and traI, 5'-CGACCAATACCAACACCAG / 5'-GTTCGAAGCGAGATCGG.

**Phylogenetic analysis of the TraR homologues.** Sequence analysis was performed using DNASTar software package and the amino acid sequences of the TraR homologues in *Rhrizobia* species were retrieved from Genbank database (http://www.ncbi.nlm.nih.gov/). Sequence alignment was performed with Clustal W (http://www.ebi.ac.uk/clustalw/) and sequence divergences were estimated with Kimura two-parameter distance (34). The phylogenetic tree was constructed using the neighbor-joining (NJ) method of PHYLIP 3.57c (35). The NJ tree was rooted using the
RhlR sequence of Pseudomonas aeruginosa as outgroup. Bootstrap analyses were carried out with 1000 replicates.

RESULTS

TraR2 is an additional regulator governing the AHL production in A. tumefaciens strain A6

We showed previously that a single amino acid mutation (L54P) in the QS antiactivator TraM is responsible for the constitutive phenotype of A. tumefaciens K588 in QS, i.e., the strain could produce AHL signals and transfer Ti plasmid in the absence of the cognate conjugal inducer octopine (25). K588 is a strain with a nopaline-type chromosomal background harboring an octopine-type Ti plasmid. To investigate the molecular basis of the QS-constitutive phenotype of A. tumefaciens K588, we introduced the L54P point mutation to the TraM of A. tumefaciens wild type strain A6 by allelic replacement to generate the strain A6(traM<sub>k588</sub>). Due to the presence of traM2 in the chromosomal background of A6, however, A6(traM<sub>k588</sub>) exhibited indistinguishable QS-related phenotypes from strain A6, including the production of AHL signals and the efficiency of Ti plasmid conjugal transfer (25). When cultured on agar plate, A6(traM<sub>k588</sub>) could produce a detectable amount of AHLs, and deletion of the second copy of traM (traM2) significantly increased the AHL production (Fig.1A), which is consistent with the previous findings (25). In this study, we exploited strain A6(traM<sub>k588</sub>, ΔtraM2) to explore additional elements involving in the QS regulation in A. tumefaciens by using A6(traM<sub>k588</sub>) as a control. Given that the AHL signals produced by A6(traM<sub>k588</sub>) in the liquid medium without addition of inducer...
octopine was not detectable (25), solid agar plates were hence used for examination of AHL production. Using A6(traM\textsubscript{k588}, ΔtraM2) as the parent strain, we carried out Tn5 transposon mutagenesis to screen for mutants with altered AHL production. Following screening for more than 10000 mutants, one was identified showing substantially decreased AHL production, which was comparable with that of A6(traM\textsubscript{k588}) (Fig.1A). Analysis of the transposon-flanking sequences showed that the transposon was inserted into an ORF encoding a traR homologue, which was designated as traR2.

To verify that the decrease of AHL production is due to the defect of traR2, we carried out the phenotypical analysis by constitutive expression of traR2 in the mutant A6(traM\textsubscript{k588}). As shown in Fig.1A, the traR2-overexpressed strain dramatically enhanced its AHL production, comparable to that of A6(traM\textsubscript{k588}, ΔtraM2), suggesting that TraR2 positively regulates the QS system in A. tumefaciens A6.

To further verify the involvement of traR2 in AHL production in A. tumefaciens A6, we transferred the low copy-number reporter vector of pLA-\textit{traG}::\textit{lacZ}, where the promoterless \textit{lacZ} fragment was transcriptionally fused with the AHL-responsive promoter of the \textit{traG} gene, into the traR2-deficient strains and then analyzed the AHL production as indicated by the β-galactosidase activity. Results showed that the β-galactosidase activity in A6(traM\textsubscript{k588}, ΔtraM2) was approximately 100-fold higher than that of A6(traM\textsubscript{k588}). However, when traR2 was mutated in this the double traM mutant, the β-galactosidase activity was considerably decreased to a level comparable to that
of the single *traM* mutant (Fig. 1B). Moreover, overexpression of *traR2* in A6(*traM*<sub>k588</sub>) dramatically enhanced the β-galactosidase activity, even higher that of the parent strain A6(*traM*<sub>k588</sub>, *ΔtraM2*) (Fig.1B). These findings are highly agreeable with the results of AHL production (Fig. 1A), supporting that *traR2* functions as a positive regulator of QS in *A. tumefaciens* A6.

In silico analysis showed that *traR2* shared about 67% and 68% identity with the *traR* of strain A6 at the nucleotide and peptide levels, respectively. The predicted secondary structures of TraR2 appear similar to that of TraR, containing ten α-helixes and five β-sheets. Sequence inspection of the N-terminal domain of TraR2 showed it shared about 61% identity with the same domain of TraR, suggesting a high degree of conservation of AHL binding sites. Among the thirteen amino acids important for ligand binding, eleven are identical between TraR and TraR2 (Fig. S1B).

Sequence inspection of the C-terminal domain also showed a high level of conservation between TraR2 and TraR of *A. tumefaciens* A6 sharing about 71% identity in amino acid sequence. However, among the ten key amino acids of TraR associated with DNA binding, TraR2 contains four varied residues (R206K, V207S, R210E and M213R) (Fig. S1B). According to the X-ray crystal structure of TraR, the side chains of Arg206 and Arg210 make base-specific interactions, forming a hydrogen bond with the bases G and T in the Tra Box, respectively; whereas the side chains of Val207 and Met213 form hydrophobic interactions with the backbone sugar of base G and base T, respectively. Considering all these four residues are purposed
to be determinants for specific recognition of TraR to its cognate DNA target, alteration
of these amino acids may indicate the variations in the DNA recognition specificity and
DNA binding affinity of these two QS regulators in *A. tumefaciens* A6.

By using chromosomal walking approach, the DNA sequences of the *traR2*-flanking
segments were determined. The sequenced segment stretched a length of ~6kb, encoding five putative ORFs. At the downstream of *traR2*, three ORFs were identified which are homologues of the *traM*, *traH* and part of *traB* of Ti plasmid, respectively, with identities ranging from 66% to 69% (Fig. S1A). Moreover, the genetic location and transcriptional orientation of these homologues appeared similar to their counterparts in Ti plasmid, suggesting a highly conserved syntenic organization. However, no obvious ORFs could be defined within the ~3kb region upstream of *traR2*, different from the case of Ti plasmid, in which *ophED*, is located at the immediate upstream of *traR* and constitute a co-transcriptional operon with *traR* and upstream genes (Fig. S1A).

**TraR2 is a functional 3OC8HSL receptor**

For further investigation of the biological roles of *traR2* in *A. tumefaciens*, the
*traR2* gene was heterogeneously and constitutively activated by the lac promoter of
pDSK in Ach5C3, an octopine-type *A. tumefaciens* strain cured of Ti plasmid and thus lacking the TraR-TraM-Trai components of the QS system carried by Ti plasmid. The AHL reporter plasmid of pLA-*traG::lacZ* was then introduced to monitor the response of TraR2 to external AHL signals. As shown in Fig. 2, when chemically synthesized
3OC8HSL was added with various concentrations, Ach5C3 and Ach5C3(pLA-traG::lacZ) both exhibited only a background level of the β-galactosidase activity. When 3OC8HSL was added to Ach5C3(pDSK-traR2, pLA-traG::lacZ), however, the β-galactosidase activity was substantially increased to a level comparable with that of the control strain NT1(pDSK-traR, pLA-traG::lacZ), in which the traR gene of Ti plasmid was constitutively expressed by the lac promoter of pDSK vector. These findings demonstrate that TraR2 alone could also serve as an AHL receptor and activate the typical QS-dependent gene expression in A. tumefaciens. Additionally, it was also noticed that the β-galactosidase activity of Ach5C3(pDSK-traR2, pLA-traG::lacZ) was much lower at low concentration of 3OC8HSL but became relatively higher at high concentration of 3OC8HSL than that of NT1(pDSK-traR, pLA-traG::lacZ), respectively (Fig. 2), suggesting that the divergence in DNA-binding domain may affect their promoter binding affinity. These results may suggest that TraR and TraR2 could play different roles under various physiological conditions of AHL signals.

**A. tumefaciens A6 contains a traI homologue traI2**

In rhizobia species, such as Rhrizobium leguminosarum, several sets of AHL-type QS systems have been identified (36). Identification of the second copies of traM and traR tempted us to assume that there may exist another copy of traI in A. tumefaciens A6. To test this assumption, we deleted the traI gene of Ti plasmid in A6(traM<sub>586</sub>) and then examined its AHL production. As shown in Fig. 4A, deletion of traI did not significantly affect the AHL production on BM solid plates, indicating the existence of another copy of traI in A. tumefaciens A6. For identification of the traI
homologue, \( A6(\text{traM}_{588}, \Delta \text{traI}) \) was used as a parent strain for Tn5 transposon mutagenesis to screen for AHL-defective mutants. Among 5000 transposon mutants screened, one was isolated as it could not produce detectable AHLs on BM solid plates (Fig. 3). As it could be expected, analysis of transposon-flanking sequences showed Tn5 inserted within a \( \text{traI} \) homologue, which was thus named as \( \text{traI}2 \). The \( \text{traI}2 \) gene encoded a peptide of 212 amino acids with a deduced molecular mass of 23.6 KDa. Sequence alignment showed that the \( \text{TraI}2 \) peptide shared about 69% identity with the peptide encoded by the \( \text{traI} \) gene of \( A. \text{tumefaciens} \) A6. Compared with \( \text{TraI} \), both ends of \( \text{TraI}2 \) appeared highly variable while the central part was relatively conserved (Fig. S2B). Similar to \( \text{traI} \) of Ti plasmid, downstream of \( \text{traI}2 \) contains the homologues of \( \text{trbB}, \text{trbC}, \text{trbD} \) and \( \text{trbE} \) respectively which likely form a co-transcriptional operon together with \( \text{traI} \). However, no typical ORFs could be found upstream of the \( \text{traI}2 \) gene, which was different from the corresponding region of Ti plasmid, in which the \( \text{rep} \) operon containing \( \text{repA}, \text{repB} \) and \( \text{repC} \) was divergently oriented at the upstream of the \( \text{traI} \) gene (Fig. S2A).

**TraI2 is a 3OC8HSL synthase**

To examine the biological function of \( \text{traI}2 \) in \( A. \text{tumefaciens} \) A6, we overexpressed the \( \text{traI}2 \) and \( \text{traI} \) genes respectively in the mutant \( A6(\text{traM}_{588}, \Delta \text{traI}, \text{traI}2::\text{Tn5}) \) and determined the AHL production on BM solid plates. As shown in Fig. 4A, the \( \text{traI}2 \)-overexpressing strain produced a large amount of AHLs, indistinguishable from that of the \( \text{traI} \)-overexpressing strain. The results demonstrate that \( \text{TraI}2 \) not only synthesized active AHLs, but also synthesized the signals as
efficiently as TraI in the background of octopine-type strain A6. Given that various active AHLs have been found in \textit{A. tumefaciens} and the solid plate-based bioassay system is not able to distinguish these signals, we employed the TLC-based assay to analysis the AHLs produced by TraI2 and TraI in \textit{A. tumefaciens} A6. The results showed that both TraI2 and TraI predominantly synthesized 3OC8HSL signals (Fig. 4B). These findings indicate that TraI2 is a functional 3OC8HSL synthase and thereby could participate in the regulation of Ti plasmid conjugal transfer.

\textbf{QS2 is independent of octopine induction}

The transcription of \textit{traR} on Ti plasmid has been well established to be induced by octopine and controlled by quorum sensing itself in the octopine-type \textit{A. tumefaciens} strains. In the presence of octopine, the transcription of \textit{traR} is activated and its product TraR forms a complex with the AHL signal 3OC8HSL. The TraR-AHL complex further increases the transcriptional expression of \textit{traI}, \textit{traM} and \textit{traR}. In this way, the QS regulatory mechanism forms a positive feedback loop triggered by octopine. The parallel roles of TraM2 and TraM, TraR2 and TraR, and TraI2 and TraI tempted us to examine the potential relationship of octopine induction and QS2 regulation. For this purpose, we firstly examined the AHL production of A6(\textit{traM}\_k588) and A6(\textit{traM}\_k588, \textit{ΔtraI}) with or without octopine induction. As shown in Fig. 5A, octopine treatment dramatically increased the AHL production of A6(\textit{traM}\_k588) but only marginally increased the AHL production by A6(\textit{traM}\_k588, \textit{ΔtraI}). These sharp contrast results didn’t seem to be favorable to the notion that QS2, which includes TraM2, TraR2 and TraI2, could be induced by octopine. For further validation, RT-PCR was
carried to study the transcriptions of the QS2-related genes in strain A6(traM<sub>k588</sub>) with or without octopine. As a control, the transcription of <i>traI</i> was dramatically enhanced with octopine treatment; in contrast, however, the transcripts levels of <i>traM2</i>, <i>traR2</i> and <i>traI2</i> did not show obvious changes regardless the presence or absence of octopine (Fig. 5B). Taken together, these data demonstrate that the regulation of QS2 is independent of octopine induction, which is different from the regulatory mechanism of the Ti plasmid-borne QS system.

**QS2 components are located on a transmissible genetic element**

In <i>A. tumefaciens</i> strains, all the QS regulatory genes are mapped on Ti plasmid and thus these components are capable of transfer among the bacterial population via Ti plasmid conjugation. The tight linkage of <i>traM2</i>, <i>traR2</i> and <i>traI2</i> with other homologues of the <i>tra</i> and <i>trb</i> genes suggested that the QS2 components may be located on a plasmid which is transmissible. To examine this hypothesis, we decided to carry out the Ti plasmid conjugation assay using A6(traM<sub>k588</sub>, Δ<i>traM2</i>, <i>traR2</i>::Tn5) as the donor strain and C58C1 as the recipient strain. The donor strain was resistant against kanamycin because the Tn5 transposon was inserted in the <i>traR2</i> gene while the recipient strain was resistant against rifampicin due to spontaneous mutation. After conjugation overnight with octopine induction, transconjugants were screened on the LB agar plates supplied with kanamycin and rifampicin. After 3-day incubation at 28 °C, single colonies appeared on the plates with a frequency of approximately 3.5 x 10<sup>-8</sup>. Three transconjugants were randomly picked up for further phenotypical characterization. To test if <i>traI2</i> was transferred into...
these transconjugants, we detected the AHL production on the plate and found that all the three transconjugants could produce detectable AHLs (Fig. 6A). PCR analysis showed that all these colonies contained the *traI2* gene. As a control, the *ska* gene, which is present in C58C1 but absent in A6, could be successfully amplified from the transconjugants (Fig. 6B), supporting that these colonies are the nopaline-type C58C1 but not the octopine-type A6. Additionally, the *traI2* and *traB2* segments, which are specific to the donor strain A6(*traM*<sub>k588</sub>, Δ*traM*<sub>2</sub>, *traR*<sub>2</sub>::Tn5) but absent in the recipient strain C58C1, could also be amplified from these colonies, indicating that the transconjugants carried the *traI2*-containing fragment (Fig. 6B). Given that the *traB2* gene is physically linked with *traM*<sub>2</sub> and *traR*<sub>2</sub> (Fig. S1A), the above results also support that the QS2 components (namely, *traM*<sub>2</sub>, *traR*<sub>2</sub> and *traI*<sub>2</sub>) may reside on a single transmissible genetic unit. It was noticed that the *traI* gene, which is unique in the donor strain, could also be amplified from one of the transconjugants, suggesting that this transconjugant also contains the Ti plasmid, which may account for the AHL-overproduction phenotype of this transconjugant (Fig. 6A). Put together, these data demonstrate that the QS2 components are located on a transmissible genetic element but its identity remains elusive at this time. Using the transconjugant carrying this transmissible element alone, we tested the potential roles of this mobile genetic element. However, the results showed that this transmissible unit neither enabled the bacterial species to utilize octopine as the sole carbon source (Fig. 6C) nor incited the crown gall tumor formation on plant host (Fig. 6D).

Using A6(*traM*<sub>k588</sub>, Δ*traM*<sub>2</sub>, *traR*<sub>2</sub>::Tn5) as the donor strain and C58C1 as the recipient strain, we also conducted conjugation assay in the absences of octopine.
However, no transconjugants were obtained on the LB agar plates containing kanamycin and rifampicin. These results suggest that octopine was essential for inducing the transmission of QS2-containing genetic element. In *A. tumefaciens* A6, octopine has been known to trigger the expression of TraR, which could directly activate the *tra2* promoters, or may activate only the *tra* promoters and subsequently the Tra proteins detect the *oriC* for transmission of the QS2-containing genetic element. Further investigations are required to distinguish these two possibilities.

**DISCUSSION**

QS has been well established as a conserved regulatory mechanism for bacterial populations to coordinately regulate gene expression. The QS of *A. tumefaciens* could be one of the best characterized paradigms of QS systems. Several proteins, including TraI, TraR, and TraM, constitute the crucial components of QS regulation in this bacterium. TraI is an enzyme that synthesizes the signal molecule 3OC8HSL, which activates TraR. The activated TraR induces the transcriptional expression of its target genes, including the *tra*, *trb*, and *rep* operons. Moreover, the transcription of *traR* is initiated by cognate conjugal opines (37). In the octopine strains, placing *tral* within the QS-responsive *trb* operon constitutes a classic QS positive feedback loop found in many Gram-negative bacteria (38). In addition, a negative feedback loop is also formed by the TraM antiactivator, whose transcription is directly activated by the QS system (16). Previous data showed that these QS regulatory genes, including the *traM*, are all located on the Ti plasmid. Recently, we identified TraM2 as an additional QS regulator which is not located on the Ti plasmid (25).
TraM2, together with its TraM parologue, dually control the Ti plasmid conjugation in *A. tumefaciens* A6. In this paper, we continued to use the A6 strain to study the QS systems in *A. tumefaciens*, and we identified TraR2 and TraI2 as additional QS regulators in this bacterial species. TraR2, like TraR, recognizes 3OC8HSL and activates the QS-responsive reporter (Fig. 2). TraI2, similar to TraI, efficiently synthesizes 3OC8HSL in *A. tumefaciens* A6 (Fig. 4). Together with TraM2 identified previously, TraR2 and TraI2 appear to constitute a second QS system (QS2) in *A. tumefaciens* A6. However, the regulation of QS2 is independent of octopine (Fig. 5), which differs from that of the Ti plasmid-borne QS system and highlighted the complexity of QS regulation in *A. tumefaciens*. The next challenges would be to unravel the molecular mechanisms involved in the QS2 regulation and to determine the extent these two QS systems could interact.

Identification of QS2 in this study also highlights the variations of QS systems in *A. tumefaciens*. Several lines of previous evidence have demonstrated the strain-specific variations of QS regulation in *A. tumefaciens*. First, plant hosts produce different opines to specifically activate the QS systems of different *A. tumefaciens* strains. For example, octopine and agrocin opine are the QS inducers for strains A6 and C58, respectively (20). Second, opine receptors may act differently in different strains. In A6, the receptor is OccR which could act as an activator. In C58, however, the receptor is AccR and only functions as a repressor. Third, the TrlR antagonist is absent in the C58 strain, while the TraM antiactivator is present only in octopine strains. Furthermore, TraM2, a TraM parologue initially identified from A6, was only identified in part of the
Identification of TraR2 and TraI2 in A6, but not in other strains such as C58, B6, K588 and R10, adds another line of evidence for the strain-specificity of the QS regulatory mechanisms in *A. tumefaciens*.

Identification of QS2 in *A. tumefaciens* is not the first example that more than one AHL-type QS systems found in a bacterial species. In *R. leguminosarum*, four QS systems have been identified and their roles are intertwined in a complex regulatory network, regulating plasmid transfer, nodulation, bacterial growth and other unknown functions (36, 37, 39, 40). In *R. etli* CNPAF512, two QS system have been found, which are involved in regulation of symbiosome development and nitrogen fixation (41, 42). In these examples, each of QS systems appeared to produce different AHL signal molecules and control different biological functions. In contrast, however, TraI2 in QS2 seemed to play an identical role with TraI of QS, both predominantly synthesizing 3OC8HSL in *A. tumefaciens* A6. Moreover, TraR2 appeared to recognize 3OC8HSL and activate the *tra* box-containing promoters as efficiently as TraR. Put together, these results strongly suggest a signaling crosstalk between QS and QS2 and we conclude that QS2 may play a redundant role with QS in regulation of Ti plasmid replication and conjugation. To date, however, we could not rule out the possibility that QS2 may also be involved in regulation of other bacterial behaviors which are independent of the QS control.

About two decades ago, we reported that octopine strains can be divided into two groups, transfer efficient (Tra<sup>e</sup>) and transfer inefficient (Tra<sup>ie</sup>), based on their Ti plasmid...
conjugal transfer efficiency (26). The Tra<sup>e</sup> group includes strains A6, NCPPB1001 and K608, whereas the Tra<sup>e</sup> group consists of B6S3, Ach5 and B6. It is interesting to note that the Tra<sup>e</sup> strain K608 is the conjugal transfer constitutive mutant (26), which is similar to the strain K588 used in this study containing a L54P point mutation in TraM, and that QS2 genes were found in the Tra<sup>e</sup> strain A6 but not in the Tra<sup>ie</sup> strains B6S3, Ach5 and B6 (25). Therefore, identification of the QS2 system in strain A6 in this study, together with the findings on the impact of L54P mutation in TraM on QS (25), seem to provide two molecular mechanisms which could affect the conjugal transfer efficiency of Ti plasmid in <i>A. tumefaciens</i>.

A cluster of genes in the symbiotic plasmid pNGR234a of <i>Rhizobium</i> sp. strain NGR234 has been found to be highly homologous to the <i>tra</i> and QS system of <i>A. tumefaciens</i> (43, 44). Given the fact that the key QS regulators, i.e., TraI, TraR and TraM, identified in this <i>Rhizobium</i> strain are the orthologues of <i>A. tumefaciens</i>, the QS of <i>Rhizobium</i> sp. strain NGR234 is proposed to regulate the transfer of pNGR234a (37). It has been demonstrated that the TraI homologue synthesizes 3OC8HSL in NGR234 (45). However, a <i>traI</i> mutant still produces an AHL signal that is likely to be 3OC8HSL, suggesting the presence of one or more additional AHL synthetases in this bacterial strain. Further analysis showed the synthetase genes should reside elsewhere in the genome, but its identity has not yet been characterized (37). Our findings that TraI2 resides outside of the Ti plasmid and predominantly produces 3OC8HSL in <i>A. tumefaciens</i> A6 may imply that strain NGR234 may also encode a TraI homologue, i.e., TraI2, which is responsible for the observed 3OC8HSL production in
its \textit{traI} mutant.

The results from this study showed that the QS2 component genes (\textit{traR2}, \textit{traI2} and \textit{traM2}) are physically linked on a transmissible genetic unit. Given that their counterparts \textit{traR}, \textit{traI} and \textit{traM} are carried by the Ti plasmid which is conjugally mobile (38), we speculated that the QS2 system genes could also be contained by a plasmid reminiscent to Ti plasmid. Using an approach as described previously for Ti plasmid isolation (26), however, we failed to isolate the putative plasmid. One possibility for this failure is that this plasmid may be too large to be isolated by routine methods, and the other possibility could be that the QS2 system is not carried in a plasmid but in an Integrative-Conjugative-Element (ICE), similar to the transmissible QS system in \textit{Serratia marcescens} (46). It has been suggested that the multiple QS systems in most bacteria appear to have been acquired from separate sources (47, 48). Further characterization of the mobile genetic element that carries the QS2 genes may shed light on the origin of QS2 system and the biological roles of this intriguing mechanism.
Figure Legend

Fig. 1. Characterization of the traR2 gene associated with AHL production in A. tumefaciens A6. (A) AHL production by A6(traMk588) and its derivatives on BM agar plates. (B) β-galactosidase activity of A6(traMk588, pLA-traG::lacZ) and its derivatives grown in BM liquid medium.

Fig. 2. Functional analysis of TraR2. The β-galactosidase activities of Ach5C3 and its relevant derivatives were determined after incubation with various concentrations of 3OC8HSL signal molecules.

Fig. 3. AHL production by A6(traMk588) and its derivatives on BM agar plate.

Fig. 4. TraI2 is an AHL synthase. (A) AHL production by A6(traMk588, ΔtraI, traI2::Tn5) and its derivatives on BM agar plate. (B) TLC analysis of the AHL molecules produced by A6(traMk588, ΔtraI, traI2::Tn5) and derivatives with chemically synthesized 3OC8HSL as a standard control.

Fig. 5. The QS2 system is independent of the octopine induction in A. tumefaciens A6. (A) AHL production by A6(traMk588) and A6(traMk588, ΔtraI) with (+) or without (-) octopine induction on BM agar plate. (B) RT-PCR analysis of traM2, traR2 and traI2 expressions with (+) or without (-) octopine induction with traI as a control. A6(pTiA6traMk588) cells were cultivated in BM medium at 28 °C degrees with and without octopine induction. Samples were collected at OD600 of 1.2 for total RNA
isolation. RT-PCR analysis was carried out using the primers described in Materials and Methods. Three concentrations of RNA templates, including an original concentration of 20 ng/µl, and its 10 × and 100 × dilutions, were used for the comparative RT-PCR analysis.

Fig. 6. The QS2 genes are located on a transmissible genetic element in *A. tumefaciens* A6. (A) AHL production by three transconjugants C1A6MR1-3 obtained after conjugal mating using A6(*traM*, A6*, traR*::Tn5) as the donor strain and C58C1 as the recipient strain. (B) PCR examination of the donor, recipient and their transconjugants. The *ska* gene, which is unique in nopaline strain C58C1 while absent in the octopine strain A6, the *tra2* and *tra* fragments, which are present in A6 but absent in C58C1, were respectively amplified by PCR. (C) Bacterial growth of various *A. tumefaciens* strains with different carbon sources as indicated in the figure. Results were recorded after 5-day growth at 28 °C degrees. (D) Tumorigenicity assay of *A. tumefaciens* on Kalanchoe leaves. Strains A6, C58C1, C1A6MR2 and C58C1(pTiA6) were grown in LB for overnight and diluted to OD₆₀₀ of 1.0. 10 µl of bacterial cultures were inoculated onto the wounded Kalanchoe leaves. The plant was grown at 24°C for 4 weeks before results recorded, and the tumors incited by A6 and C58C1(pTiA6) are highlighted in red circles.
Table 1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics a</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>A. tumefaciens</td>
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<tr>
<td>A6</td>
<td>The wild type octopine strain of <em>A. tumefaciens</em></td>
<td>A. Kerr</td>
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<tr>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;)</td>
<td>A6 with <em>traM</em> replaced by <em>traM</em> of strain K588 which is dysfunctional</td>
<td>(25)</td>
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<tr>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;,ΔtraM2)</td>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;) carrying the inframe deletion of <em>traM</em></td>
<td>(25)</td>
</tr>
<tr>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;,ΔtraM2;</td>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;) carrying the Tn5 insertion of <em>traR</em>, Kan&lt;sup&gt;′&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>traR2::Tn5)</td>
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<tr>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;,ΔtraM2,</td>
<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;) carrying the AHL-responsive reporter</td>
<td>This study</td>
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<td>pDSK-traR2)</td>
<td>pLA-traG::lacZ, Kan&lt;sup&gt;′&lt;/sup&gt; and Tc&lt;sup&gt;′&lt;/sup&gt;</td>
<td>(9)</td>
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<td>C58C1</td>
<td>Derivative of the nopaline strain C58 cured of the Ti plasmid used</td>
<td>SK Farrand</td>
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<td>for conjugation analysis, Rif&lt;sup&gt;′&lt;/sup&gt;</td>
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<td>A6(traM&lt;sup&gt;K588&lt;/sup&gt;,ΔtraI)</td>
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<td>traI2::Tn5)</td>
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<td>C1A6MR1</td>
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C1A6MR2
Transconjugant of A6(traM_{A6}, Δ traM2; traR2::Tn5) and C58C1, Rifr and Kanr
This study

C1A6MR3
Transconjugant of A6(traM_{A6}, Δ traM2; traR2::Tn5) and C58C1, Rifr and Kanr
This study

CS8C1(pTiA6)
C58C1 carrying the Ti plasmid of A6
(25)

E. coli

DH5α (λpir)
supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1, λpir
Laboratory collection

BW020767(pRL27)
Harbouring Tn5 for A. tumefaciens mutagenesis
(49)

Plasmids

pK18mobsacB
The broad-host-range gene replacement vector; Sucr and Kanr

pK18-traI
pK18mobsacB harboring a deleted traI-flanking region, Kanr
This study

pLAFR3
IncP, broad host range cosmid vector, Tc r

pLA-traG::lacZ
pLAR3 carrying the traG gene transcriptionally fused with promoterless lacZ, Tc r
(9)

pLA-traI
pLAFR3 harboring the traI gene from A6, Tc r
This study

pLA-traI2
pLAFR3 harboring the traI2 gene from A6, Tc r
This study

pDSK
IncQ, broad host range plasmid, Kanr

pDSK-traR
pDSK harboring the traR gene from A6, Kanr
(9)

pDSK-traR2
pDSK harboring the traR2 gene from A6, Kanr
This study

**Abbreviations:**
- Ampr, ampicillin resistant; Kanr, kanamycin resistant; Tc r, tetracycline resistant;
- Rifr, rifampicin resistant, Suc r, sucrose resistant.
Reference 572


43. **Farrand SK, Hwang I, Cook DM.** 1996. The tra region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J Bacteriol 178:4233-4247.


Fig. 1

A

B

β-galactosidase activity (U/10^9 CFU)

- A6(traM/cys)
- A6(traM/cys) tmaM2
- A6(traM/cys) pDSK-traR2
- A6(traM/cys) pDSK-traR2

Note: The figure illustrates the β-galactosidase activity of different strains and constructs, with the y-axis representing activity in U/10^9 CFU and the x-axis showing different genetic backgrounds.
Fig. 2

β-galactosidase activity (U/10^9 CFU)

0 200 400 600 800 1000

Control
1nM COOH
100nM COOH
Fig. 3
Fig. 4

A

B
Fig. 5

A  

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<tr>
<td>A6(traM&lt;sub&gt;est&lt;/sub&gt;, ΔtraI)</td>
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B  

- octopine  

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<td>16S rDNA</td>
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RNA template dilution times: 1x, 10x, 100x
Fig. 6

A

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B

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<th>M</th>
<th>A6(tralM::&lt;sup&gt;cre&lt;/sup&gt;)</th>
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<td>C58C1</td>
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<tr>
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<tr>
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<td>3</td>
<td>tral</td>
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</table>

C

- No bacterial inoculum
- No carbon source
- Octopine as carbon source
- Mannitol as carbon source

D

- C58C1(pA6Ti)
- C1A6MR2

- A6
- C58C1