4	
т	

3	Title: PsyR, a transcriptional regulator in quorum sensing system, binds <i>lux</i> box-like
4	sequence in <i>psyI</i> promoter without AHL quorum sensing signal molecules and activates
5	psyI transcription with AHL in Pseudomonas syringae pv. tabaci 6605
6	i) Authors' names: Yuki Ichinose ^{1,2,*} , Yousuke Tasaka ¹ , Satoru Yamamoto ¹ , Yuko Inoue ² ,
7	Motohiro Takata ¹ , Yukiko Nakatsu ¹ , Fumiko Taguchi ^{1,3} , Mikihiro Yamamoto ^{1,2} , Kazuhiro
8	Toyoda ^{1,2} , Yoshiteru Noutoshi ^{1,2} and Hidenori Matsui ^{1,2}
9	ii) Affiliations and addresses: ¹ Graduate School of Environmental and Life Science,
10	Okayama University, 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530 Japan. ² Faculty
11	of Agriculture, Okayama University, 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530,
12	Japan. ³ Present address: Department of Biotechnology, Graduate School of Engineering,
13	Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan
14	iii) *For correspondence: E-mail <u>yuki@okayama-u.ac.jp</u> ; Tel. and FAX (+81) 86 251 8308.
15	
16	Number of figures and tables: 6 figures and 2 tables (4 supplemental figures)
17	Word count: 5434 (text) and 602 (Tables)
18	Running title: AHL-mediated quorum sensing in Pseudomonas syringae
19	

1 **Abstract** (248 words)

2 Quorum sensing (QS) is a mechanism for bacterial cell–cell communication using QS signals. 3 *N*-acyl-homoserine lactones (AHL), QS signals in *Pseudomonas syringae* pv. *tabaci* (*Pta*) 4 6605, are synthesized by an AHL synthase (PsyI) and recognized by its cognate transcription 5 factor PsyR. To reveal the role of PsyR in virulence, we generated $\Delta psyR$ mutant and 6 complemented strains in *Pta* 6605. Thus, we found that the $\Delta psyR$ mutant is remarkably 7 reduced in AHL production and ability to cause disease and propagate in host tobacco leaves. 8 The phenotypes of complemented strains were restored to that of the wild-type (WT). 9 Because the $\Delta psyR$ mutant lost nearly all AHL production, we investigated the function of 10 PsyR in the transcription of *psyI* and AHL production. Electrophoretic mobility shift assays 11 suggested that the recombinant PsyR protein binds the promoter region of *psyI* but not *psyR* 12 without AHL. The addition of AHL did not significantly affect this binding. The binding core 13 sequence of this region was identified as a 20 bp *lux* box-like sequence. To reveal the 14 function of PsyR and AHL on *psyI* transcription, we constructed a *psyI* promoter::*lacZYA* 15 chimeric reporter gene, and transformed it into the WT and $\Delta psyI$ mutant of *Pta* 6605. The 16 β-galactosidase activity was increased in a bacterial density-dependent manner in the WT and 17 also in a $\Delta psyI$ mutant by the addition of exogenous application of AHL. These results 18 indicate that the solo PsyR binds the *lux* box in the *psyI* promoter and activates transcription 19 in the concomitant presence of AHL.

20

21 Key words: AHL; PsyI; PsyR; quorum sensing

22

1 Introduction

2 Quorum sensing (QS) is a system for bacterial cell-cell communication using small diffusible 3 signal molecules, which are also called QS signals or autoinducers. Many Gram-negative 4 bacteria such as members of the genera Erwinia, Vibrio, Pantoea, and Pseudomonas use 5 *N*-acyl-homoserine lactones (AHL) as QS molecules (Ham 2013; Tsai and Winans 2010; von 6 Bodman et al. 2003). AHLs are synthesized by the coupling of homoserine lactone rings from 7 S-adenosylmethionine and acyl chains from the acyl-acyl carrier protein (acyl-ACP) pool in 8 cells by the AHL synthase enzymes, such as LuxI in Vibrio fischeri (Whitehead et al. 2001), 9 EsaI in Pantoea stewartii (Beck von Bodman and Farrand 1995), and PsyI (or also called 10 Ahll or PsmI) in *Pseudomonas svringae* (Elasri et al. 2001; Taguchi et al. 2006; Ouiñones et 11 al. 2004). N-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) is one of the most commonly 12 reported molecules of quorum sensing signals. The concentration of OHHL increases as 13 bacterial density increases. Thus, in V. fisheri, the molecular complex of transcription factor 14 LuxR with OHHL binds to a promoter of the *luciferase* operon, *luxI*, which encodes AHL 15 synthase and bioluminescence-related proteins, and activates its transcription when the 16 concentration of OHHL reaches a threshold (Urbanowski et al. 2004). QS controls a wide 17 variety of bacterial behaviors such as bioluminescence, swarming motility, biofilm formation, 18 multidrug resistance, production of extracellular enzymes, antibiotics, epiphytic fitness, and 19 altogether affects bacterial virulence (Fuqua et al. 2001; von Bodman et al. 2003). 20 In the best known QS system in V. fischeri, LuxR-type transcription factors dimerize 21 and with AHL the complex binds to target promoters to activate transcription. However, it is 22 also reported that another LuxR-type transcription factor, EsaR of Pantoea stewartii, a 23 causative agent of Stewart's wilt of sweet corn and leaf blight of maize, negatively controls 24 its own transcription, but its cognate AHL synthase gene *esal* is not regulated by EsaR (von 25 Bodman et al. 2003). EsaR proteins dimerize, bind the promoter region of esaR itself and the 26 genes involved in exopolysaccharide (EPS) production in the absence of AHL to inhibit

1 transcription as a repressor (Beck von Bodman and Farrand 1995; Carlier et al. 2009; 2 Minogue et al. 2002). However, it was reported that EsaR in the absence of AHL also 3 functions as a transcriptional activator for small regulatory RNA, esaS (Schu et al. 2009). The 4 EsaR controls divergently transcribed genes *esaR* and *esaS*. The EsaR binding site is located 5 at a position centered around -10 of esaR and a position centered around -60 of esaS. EsaR 6 binding in the absence of AHL repressed transcription of *esaR*, but activated that of *esaS*. 7 Therefore, EsaR is an activator when binding site located at a position around -60, whereas 8 EsaR is a repressor when binding site located at a position around -10. In the presence of 9 AHL EsaR was released from the DNA, and the transcription of esaR was derepressed and 10 that of esaS was silenced (Schu et al. 2009; Shong et al. 2013; von Bodman et al. 2003). Thus, 11 the function of EsaR is changed by the binding site in the promoter of target genes and the 12 existence of AHL as a transcriptional activator and repressor. 13 It is also known that YenR, quorum sensing transcription factor in Yersinia 14 enterocolitica, activates transcription of small noncoding RNA gene, yenS, and the yenS 15 RNA involves the regulation of AHL production (Tsai and Winans 2011). In both P. stewartii 16 and Y. enterocolitica a set of genes, esaI and esaR, and yenI and yenR, are divergently 17 transcribed. Furthermore, esaS in P. stewartii and yenS in Y. enterocolitica lie adjacent to and 18 divergent from each esaR and yenR, respectively. 19 *P. syringae* is a Gram-negative bacterial pathogen that causes leaf spot, stem canker on 20 a wide range of plant species. Species of P. syringae is subdivided into about 50 pathogen 21 varieties (pathovars) based on their host plant species and type of disease symptoms. 22 Multilocus sequence analysis revealed that *P. syringae* species were divided into five clades 23 (Studholme 2011). Role of QS and regulation of AHL biosynthesis in P. syringae were 24 mainly investigated in P. syringae pv. syringae (Pss) B728a, a causal agent of brown spot on 25 bean which belongs to clade 2b (Quiñones et al. 2004) and P. syringae pv. tabaci (Pta) 6605, 26 a causal agent of tobacco wild fire disease which belongs to clade 3 (Kawakita et al. 2012;

1 Marutani et al. 2008; Taguchi et al. 2006). Pss B728a produces mainly OHHL as QS molecule by AHL synthases AhlI, the product of *ahlI*, whereas *Pta* 6605 produces OHHL and 2 3 *N*-hexanoyl-L-homoserine lactone (HHL) as major QS molecules by AHL synthase PsyI, the 4 product of *psyI*. It was thought that each LuxR type transcription factor AhlR in *Pss* B728a 5 and PsyR in *Pta* 6605 together with AHL regulates each transcription of *ahll* and *psyl*, 6 respectively. A *psyI* and *ahlI* genes, and *psyR* and *ahlR* genes are orthologues each other, and 7 amino acid identities of PsyR and AhlR, and PsyI and AhlI are high at 93% and 84%, and 8 their similarities are 100% and 98%, respectively. Both set of genes, *psyI* and *psyR* or *ahlI* 9 and *ahlR*, are transcribed convergently and responsible for AHL production as well as *esaI* 10 and esaR in P. stewartii and yenI and yenR in Y. enterocolitica. However, there is a neither 11 similar sequence to esaS of P. stewartii nor venS of Y. enterocolitica. Thus, transcriptional 12 regulation of *psyI* by PsyR or *ahlI* by AhlR remains unclear. 13 In Pss B728a, expression of ahll increased in a cell-density-dependent manner, and the 14 expression of *ahlI* was remarkably reduced in both *gacA* and *aefR* mutants (Quiñones et al. 15 2004). The GacS/GacA two-component system is one of perception system for environmental 16 signals in Gram-negative bacteria (Newton and Fray, 2004). The GacS is a sensor kinase 17 which autophosphorylate upon signal perception, and activates its partner, response regulator 18 GacA. Whereas, TetR family transcription factor, AefR functions as AHL production and 19 epiphytic fitness regulator (Quiñones et al. 2004). We previously investigated AHL 20 production-deficient mutant strains in Pta 6605 such as a psyI-defective mutant (Taguchi et al. 21 2006), Gac two-component-system defective mutants (Marutani et al. 2008), and a $\Delta aefR$ 22 mutant (Kawakita et al. 2012). Furthermore, we found that flagellar motility-defective mutant 23 strains such as $\Delta fliC$ (Taguchi et al. 2010) and $\Delta motABCD$ (Kanda et al. 2011) also impaired

24 AHL production. Recently, we found that the MexEF-OprN multidrug efflux pump

transporter negatively controls AHL accumulation in *Pta* 6605 (Sawada et al. 2018).

26 However, it is not known how *psyI* transcription is regulated in *P. syringae* species. As a first

1 step to revealing the mechanism of AHL production and the QS system in *P. syringae*, we 2 generated a $\Delta psyR$ mutant in *Pta* 6605 and examined its virulence. We further produced a 3 recombinant PsyR protein, and the specific binding of PsyR to target DNA was investigated. 4 Finally, we generated a system for monitoring *psyI* transcription using a *lacZYA* reporter gene, 5 and measured cell density-dependent and AHL-regulated *psyl* transcription. Based on these 6 analyses we discuss the role of PsyR in the regulation of gene expression and bacterial 7 virulence. 8 9 Materials and methods 10 Bacterial strains and growth conditions

11 All bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas*

12 syringae pv. tabaci 6605 strains were maintained in King's B (KB) medium at 27°C, and

13 Escherichia coli strains were grown at 37°C in Luria–Bertani (LB) medium.

14 Chromobacterium violaceum CV026 was grown at 30°C in LB medium with kanamycin at a

15 final concentration of 50 μg/ml (McClean *et al.* 1997).

16

17 Plant material, inoculation procedure and bacterial growth

18 Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi NC) were grown at 25°C with a 12 h

19 photoperiod. For dip-inoculation, bacterial strains were suspended in 10 mM MgSO₄ and

20 0.02% (v/v) Silwet L77 (OSI Specialties, Danbury, CT) at an OD₆₀₀ of 0.1, and detached

21 tobacco leaves (three leaves for each strain) were soaked in that solution for 20 min. The

22 leaves were incubated under conditions of 85% humidity in a growth cabinet for 11 days at

23 23°C with an 18 h photoperiod. To maintain the turgidity of the detached leaves, water was

supplied to the cut petiole. To examine the bacterial growth in inoculated tobacco leaves, five

25 leaf disks (8 mm diameter) were punched from tobacco leaves and soaked in a bacterial

suspension in 10 mM MgSO₄ and 0.02% (v/v) Silwet at an OD₆₀₀ of 0.1 for 20 min. The disks

were soaked in 15% (v/v) H₂O₂ for 1 min to sterilize the leaf surface 2 and 6 days after
 inoculation, then were washed with sterile distilled water and ground in a mortar with a pestle.
 Serially diluted samples (10 µl) in 10 mM MgSO₄ were spotted on KB plates, and the
 numbers of colonies that appeared after 48 h incubation at 27°C were counted, and the
 bacterial populations were calculated.

6

7 Generation of *psyI* and *psyR* mutant strains and *psyR*-complemented strain

8 To generate $\Delta psyR$ mutant of *Pta* 6605, each upstream and downstream region of *psyR* was 9 amplified with sets of primers, psyR-5F and psyR-5R for the upstream region and psyR-3F 10 and psyR-3R for the downstream region, by PCR using genomic DNA as a template (Fig. 1a, 11 Table 2). Each PCR-amplified DNA fragment was digested at the artificial BamHI site 12 designed at the 5' end of psyR-5R and psyR-3F. After ligation of the appropriate upstream 13 and downstream fragments, PCR was carried out with psyR-5F and psyR-3R primers to 14 amplify ca. 1.8 kb DNA fragment, then inserted into a pCR Blunt II TOPO vector (Thermo 15 Fisher Scientific, Waltham, MA, USA) to obtain pCR Blunt- $\Delta psyR$. The DNA fragment 16 possessing both upstream and downstream regions of *psyR* was excised by *Eco*RI digestion 17 and inserted into a mobilizable cloning vector pK18mobSacB (Schäfer et al. 1994) to obtain 18 pK18- $\Delta psyR$. On the other hand, to generate the $\Delta psyI$ mutant, psyI and its surrounding 19 region was amplified by PCR using a set of PCR primers, psyI-1 and psyI-2 (Table 2); dAMP 20 (deoxyadenosine monophosphate) was added to the 3' end of the PCR product with $10 \times$ 21 A-attachment mix (Toyobo, Osaka, Japan), and then inserted into a pGEM-T Easy vector 22 (Promega, Madison, WI, USA). Using a recombinant plasmid DNA, pGEM-*psvI*, as a 23 template, inverse PCR was carried out using a set of PCR primers, psyI-3 and psyI-4, to 24 delete the *psyI* open reading frame. Then the PCR product and template DNA were digested 25 with XbaI and DpnI. The resultant DNA was self-ligated with a Ligation-convenience kit 26 (Nippon Gene, Tokyo, Japan). The $\Delta psyI$ DNA construct was introduced into the *Eco*RI site

of pK18*mobsacB* to obtain pK18-Δ*psyI*. The pK18-Δ*psyR* and pK18-Δ*psyI* were introduced
 into *E. coli* S17-1, and the Δ*psyR* and Δ*psyI* deletion mutants of *Pta* 6605 were generated by
 conjugation and homologous recombination according to the method described previously
 (Taguchi et al. 2006).

5 For complementation of the $\Delta psyR$ mutant, primers psyR-1 and psyR-2 (Fig. 1a, Table 6 2) were used to amplify the predicted promoter region and entire *psvR* ORF, and cloned in 7 pGEM-T Easy vector to obtain pGEM-psyR. The cloned DNA fragment was excised by 8 *Eco*RI digestion, and inserted into the *Eco*RI site of pBSL118, a transposon vector (Alexeyev 9 et al. 1995), to generate pBSL-*psyR*, and introduced into the $\Delta psyR$ mutant by conjugation 10 using the E. coli S17-1 λ pir strain to generate psyR-C. All sequences of amplified DNA 11 fragments were determined by an ABI PRISM 3100 using a BIG Dye terminator cycle 12 sequencing kit (Thermo Fisher Scientific).

13

14 Detection of *N*-acyl homoserine lactones

Bacterial strains were grown in LB medium with 10 mM MgCl₂ or KB medium for 24 h at
27°C. AHLs extracted with an equal volume of ethyl acetate were detected using C₁₈
reversed-phase thin layer chromatography (TLC Silica gel 60, Merck, Darmstadt, Germany)
and the biosensor *C. violaceum* CV026 (Taguchi et al. 2006). As the standard molecules,
chemically synthesized *N*-hexanoyl-L-homoserine lactone (HHL, Sigma-Aldrich, St. Louis,
MS, USA) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL, Sigma-Aldrich) were used.

22 Production of recombinant PsyR protein

23 To produce recombinant PsyR protein (rPsyR), the *psyR* coding region was amplified with a

set of PCR primers (psyR-F and psyR-R, Table 2), and inserted into pMALc5X-His (New

- 25 England Biolabs, Ipswich, MA, USA) at NdeI and EcoRI sites to generate pMAL-psyR. The
- 26 recombinant plasmid pMAL-psyR was introduced into E. coli BL21, and rPsyR was

1 produced according to the manufacturer's instructions as fusion protein with maltose binding

2 protein (MBP). The rPsyR was further purified using a column filled with amylose resin.

3 Purified rPsyR was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

4

5 Electrophoretic mobility shift assay of PsyR

6 The interaction of rPsyR with the upstream region of *psyI* or *psyR* was assessed by an

7 electrophoretic mobility shift assay (EMSA). DNA fragments were prepared by PCR with

8 sets of primers (psyI-PF and psyI-PR for *psyI* and psyR-PF and psyR-PR for *psyR*) or

9 annealing of sets of oligonucleotides (Table 2), and DIG-labeled according to the DIG Gel

10 Shift Kit 2nd Generation (Sigma-Aldrich). An EMSA reaction mixture (20 µl) was prepared

11 according to the manufacturer's instructions. After electrophoresis, protein-DNA complexes

12 were transferred onto a Hybond-N+ membrane (GE Healthcare Biosciences, Piscataway, NJ,

13 USA), and detected with an anti-DIG antibody conjugated with alkaline phosphatase (Roche,

14 Basel, Switzerland) and CSPD as a chemiluminescence substrate (Thermo Fisher Scientific).

15 The chemiluminescence was detected with ChemiDoc Touch (Bio-Rad Laboratories,

16 Hercules, CA, USA).

17

18 Construction of reporter plasmid

19 A transposon vector pBSL118 digested at *Eco*RV and a general-purpose plasmid vector

20 pHSG396 (Takara Bio, Kusatsu, Japan) digested at *Hin*cII were ligated to produce pBSLC2,

21 then the *lacZYA* fragment was excised from pARO-*lacZYA* (Tamura et al. 2005) by *Bam*HI

and *Eco*RI digestion and inserted into pBSLC2 to create pBSLC2-*lacZYA* (Fig. 1b). The *psyI*

23 promoter region obtained by PCR with a set of primers psyI-F and psyI-R (Table 2) was

24 digested with *Bam*HI and *Spe*I, and inserted into the same restriction sites of pBSLC2-*lacZYA*

to generate pBSLC2-*psyI-lacZYA*.

26

1 β-galactosidase activity to monitor *psyI* expression

The β-galactosidase activity was measured as described by Miller (1992) with some
modification. The *psyl::lacZYA* chimeric reporter gene was introduced into a WT strain to
investigate cell density-dependent AHL production and Δ*psyl* strain to investigate the effect
of exogenous application of AHL; then β-galactosidase was measured. Promoter-less *lacZYA*was introduced into WT as a negative control. Values are expressed as the means of at least
three independent experiments.

8

9 **Results and Discussion**

10 **Phenotype of** $\Delta psyR$ **mutant**

The AHL production in the WT, Δ*psyR* mutant, and its complemented strain was examined
using *C. violaceum* CV026, the aforementioned AHL biosensor strain (Taguchi et al. 2006).
The Δ*psyR* mutant strain greatly reduced the level of AHL production, but the complemented
strain restored it to the WT level (Fig. 2).

15 To investigate the virulence of the $\Delta psvR$ mutant strain, we carried out dip-inoculation 16 using tobacco leaves with WT, the $\Delta psyR$ mutant, and its complemented strain psyR-C of Pta 6605. As shown in Fig. 3a, the disease symptoms developed in the inoculation with WT and 17 18 *psyR*-C strains, whereas severe symptoms did not appear after inoculation with the $\Delta psyR$ 19 mutant strain. The bacterial growth in tobacco leaf disks was consistent with the development 20 of disease symptoms, and the population of the $\Delta psyR$ mutant was lower than that of other 21 strains at 2 and 6 days after inoculation (Fig. 3b), although there is no significant difference 22 in their in vitro growth (Fig. S1). These results clearly suggested that the virulence of the 23 $\Delta psyR$ mutant was remarkably reduced, but that of the psyR-C strain was recovered. Thus, 24 AHL-mediated quorum sensing system is important for virulence in *Pta* 6605. This result is 25 consistent with the previous result that the $\Delta psyI$ mutant had remarkably reduced virulence 26 (Taguchi et al. 2006).

1

2 Specific binding of recombinant PsyR to a promoter of *psyI*

3 The rPsyR protein was produced using E. coli, and specific binding to the promoter of 4 potential target genes, *psyI* and *psyR*, was investigated by electrophoretic mobility shift assay 5 (EMSA, Fig. 4a). Purified rPsyR (280 nM or 480 nM) was incapable of shifting the promoter 6 of *psvR*; however, shifted bands (C1 and C2) appeared in the promoter of *psvI*. Although, the 7 identity of the two shifted bands is not clear, we expect that C1 seems to be composed of one 8 rPsyR and DNA, and C2 seems to be composed of two rPsyR and DNA. Because most 9 members of LuxR protein family function as dimer (Stevens et al. 2011). The dissociation 10 constant (Kd) of the PsyR for psyI promoter was roughly estimated as 70 nM (Fig. S2). To 11 investigate the effect of AHL on binding, different concentrations and combinations of 12 OHHL and HHL were mixed in a binding mixture of rPsyR and *psyI* promoter (Fig. 4b). 13 However, addition of AHL did not abolish the binding and had no apparent effect. Because 14 the rPsyR protein contains MBP at N-terminus, it is possible that MBP interfered the access 15 of AHL to the original N-terminal domain of PsyR which has AHL binding properties (Fuqua 16 et al., 2001). Although our attempt to purify intact rPsyR protein by removing MBP domain 17 was failed, we could generate the $\Delta psyR$ strains which possess fusion gene for MBP and 18 PsyR. The complemented strains by fusion gene for MBP/psyR evidently restored AHL 19 production (Fig. S3), indicating that the fusion protein MBP/PsyR is functional in nature. 20 Therefore, it is evident that PsyR without AHL bound to the promoter of psyI. AHL might 21 make binding stronger or change the conformation of PsyR to transcriptionally active form. 22 In the promoter region of the genes that are regulated by quorum sensing, *lux* box-like 23 sequences are conserved as binding sites for transcriptional regulators such as LuxR. The lux 24 box was first identified in the promoter of *luxI* in V. fischeri ATCC7744 (Devine et al., 1988). 25 The lux box-like sequences consist of 20 bp (core in Fig. 5) and are imperfect inverted 26 repeats. The lux box-like sequences also found in the esaR promoter of Pantoea stewartii

1 (Minogue et al., 2002), psmI promoter of P. syringae pv. maculicola strain CFBP 10912-9 2 (Elasri et al., 2001), and psyI promoter of Pta 6605 (Sawada et al. 2018, Fig. 5b). To confirm 3 the specific binding of PsyR to the *lux* box-like sequence, an EMSA competition assay was 4 carried out. A 153 bp fragment of the psyl promoter was DIG-labeled, and the lux box-like 5 sequence-containing fragments and related DNA fragments were used as competitors (Fig. 6 5a). As shown in Fig. 5c, addition of the *lux* box-like sequence containing DNA fragments 7 (60 and core) to an EMSA reaction mixture competed completely, and shifted bands 8 disappeared. However, this competition was not found by the addition of the DNA fragments 9 that did not contain *lux* box-like sequence (5'-20 and 3'-20, Fig. 5c). Although a slight 10 competition was observed by the addition of 60m, it might be due to the mutated sequence of 11 60m (5 repeats of ACGT that is possible to make inverted repeat). These results clearly 12 suggest that the *lux* box-like sequence plays a central role in this binding.

13

14 Transcriptional regulation of *psyI* promoter by PsyR and AHL

15 Incubation of *Pta* 6605 WT possessing a chimeric reporter gene *psyl::lacZYA* was started at a 16 variety of cell densities ($OD_{600} = 0.01, 0.1, 0.5, and 1.0$), and the β -galactosidase activities 17 were measured after 4 h. As shown in Fig. 6a, the β -galactosidase activity was low when 18 bacteria started incubation at 0.01 or 0.1 cell densities, whereas it became higher at 0.5 or 1.0 19 cell densities. Although high cell density activated β -galactosidase activity, this effect was 20 nearly saturated at 0.5 cell density. The *psyI*::*lacZYA* was also introduced into $\Delta psyI$ mutant. 21 The β -galactosidase activity of the $\Delta psyl$ mutant was low without application of AHL, but it 22 increased with the application of AHL in a dose-dependent manner (Fig. 6b). These results 23 indicate that *psyI* transcribed at a constitutively low level without AHL, but it will be 24 activated with AHL.

We observed that the *psyR* gene expression in the Δ*psyI* mutant was reduced to 1/3
level in wild type strain (Taguchi et al. 2015). The *psyR* gene expression decreased as well as

psyI in other AHL production-defective mutants such as Δ*fliC*, Δ*fliD*, Δ*motABCD*, Δ*fgt1* and
 Δ*aefR* (Taguchi et al. 2015). Although it is reported that transcription of *psyI* and *psyR* are
 independently regulated in *Pss* B728a (Quiñones et al. 2004), expression of *psyR* is high
 when *psyI* is actively transcribed and low when not (Taguchi et al. 2015). Further, exogenous
 application of AHL did not induce *psyI* expression in Δ*psyR* mutant (Fig. S4). These results
 indicate that transcription of *psyI* requires PsyR and AHL, and transcription of *psyI* and *psyR*

8 The LuxR protein family was subdivided into five different classes based on 9 interactions of the LuxR homologues with AHL, DNA-binding activity, and their multimeric 10 properties (Stevens et al. 2011). Among them, classes 1–3, including LuxR type transcription 11 factors, are transcriptional activators; for example, LuxR was reported to bind the *luxI* 12 promoter with AHL (Urbanowski et al. 2004), class 4, such as EsaR type transcriptional 13 regulators, bind target DNA without AHL as repressors, and class 5 regulators do not 14 dimerize in response to AHL, and the AHL they recognize is produced from neighboring 15 cells; there is no partner LuxI homologue. PsyR of *Pta* 6605 is similar to classes 1–3 as a 16 transcriptional activator. However, unlike classes 1–3, PsyR binds its target DNA without AHL, and activates transcription with AHL. Therefore, PsyR belongs to a novel class of 17 18 transcriptional regulator. Recently, we reported that MexEF-OprN, a multidrug efflux pump 19 transporter, negatively regulates AHL accumulation (Sawada et al. 2018). In this research 20 process, we also clarified that a *lux* box-like sequence is essential for the activation of *psyI* 21 transcription, because the mutant of *Pta* 6605 with *lux* box-deleted *psyI* promoter is impaired 22 in AHL production. This result also supports the hypothesis that PsyR is a transcriptional 23 activator and the *lux* box is a positive element for *psyI* transcription.

24

25 Acknowledgments

1	We would like to thank the Leaf Tobacco Research Laboratory of Japan Tobacco Inc. for
2	providing Pta 6605. This work was supported in part by Grants-in-Aid for Scientific
3	Research (No. 15H04458 and 19H02956) from the Ministry of Education, Culture, Sports,
4	Science and Technology of Japan.
5	
6	Compliance with ethical standards
7	Conflicts of interest
8	The authors declare that they have no conflict of interest.
9	Ethical approval
10	This article does not contain any studies with human participants or animals performed by
11	any of the authors.
12	
13	References
14	Alexeyev MF, Shokolenko IN, Croughan TP (1995) New mini-Tn5 derivatives for insertion
15	mutagenesis and genetic engineering in gram-negative bacteria. Can J Microbiol
16	41:1053-1055
17	Beck von Bodman S, Farrand SK (1995) Capsular polysaccharide biosynthesis and
18	pathogenicity in Erwinia stewartii require induction by an N-acylhomoserine lactone
19	autoinducer. J Bacterial 177:5000-5008
20	Carlier A, Burbank L, von Bodman SB (2009) Identification and characterization of three
21	novel EsaI/EsaR quorum-sensing controlled stewartan exopolysaccharide biosynthetic
22	genes in Pantoea stewartii ssp. stewartii. Mol Microbiol 74:903-913
23	Devine JH, Vountryman C, Baldwin TO (1988) Nucleotide sequence of the <i>luxR</i> and <i>luxI</i>
24	genes and structure of the primary regulatory region of the lux regulon of Vibrio fischeri
25	ATCC 7744. Biochemstry 27:837-842

1	Elasri M, Delorme S, Lemanceau P, Stewart G, Laue B, Glickmann E, Oger PM, Dessaux Y
2	(2001) Acyl-homoserine lactone production is more common among plant-associated
3	Pseudomonas spp. than among soilborne Pseudomonas spp. Appl Environ Microbiol
4	67:1198-1209
5	Fuqua C, Parsek MR, Greenberg EP (2001) Regulation of gene expression by cell-to-cell
6	communication: acyl-homoserinelactone quorum sensing. Annu Rev Genet 35:439-468
7	Ham JH (2013) Intercellular and intracellular signalling systems that globally control the
8	expression of virulence genes in plant pathogenic bacteria. Mol Plant Pathol 14:308-322
9	Kanda E, Tatsuta T, Suzuki T, Taguchi F, Naito K, Inagaki Y, Toyoda K, Shiraishi T,
10	Ichinose Y (2011) Two flagellar stators and their roles in motility and virulence in
11	Pseudomonas syringae pv. tabaci 6605. Mol Genet Genomics 285:163-174
12	Kawakita Y, Taguchi F, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y (2012)
13	Characterization of each <i>aefR</i> and <i>mexT</i> mutant in <i>Pseudomonas syringae</i> pv. <i>tabaci</i>
14	6605. Mol Genet Genomics 287:473-484
15	Marutani M, Taguchi F, Ogawa Y, Hossain MM, Inagaki Y, Toyoda K, Shiraishi T, Ichinose
16	Y (2008) Gac two-component system in Pseudomonas syringae pv. tabaci is required
17	for virulence but not for hypersensitive reaction. Mol Genet Genomics 279:313-322
18	McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH,
19	Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and
20	Chromobacterium violaceum: exploitation of violacein production and inhibition for the
21	detection of N-acylhomoserine lactones. Microbiology 143:3703-3711
22	Miller JH (1992) The lac system. In: Miller JH (ed) A Short Course in Bacterial Genetics. A
23	Laboratory Manual and Handbook for Escherichia coli and Related Bacteria. Cold
24	Spring Harbor Laboratory Press, Plainview, pp 43-80

1	Minogue TD, Wehland-von Trebra M, Bernhard F, von Bodman SB (2002) The
2	autoregulatory role of EsaR, a quorum-sensing regulator in Pantoea stewartii ssp.
3	stewartii: evidence for a repressor function. Mol Microbiol 44:1625-1635
4	Newton JA, Fray RG (2004) Integration of environmental and host-derived signals with
5	quorum sensing during plant-microbe interactions. Cell Microbiol 6:213-224
6	Quiñones B, Pujol CJ, Lindow SE (2004) Regulation of AHL production and its contribution
7	to epiphytic fitness in Pseudomonas syringae. Mol Plant-Microbe Interact 17:521-531
8	Sawada T, Eguchi M, Asaki S, Kashiwagi R, Shimomura K, Taguchi F, Matsui H, Yamamoto
9	M, Noutoshi Y, Toyoda K, Ichinose Y (2018) MexEF-OprN multidrug efflux pump
10	transporter negatively controls N-acyl-homoserine lactone production in Pseudomonas
11	syringae pv. tabaci 6605. Mol Genet Genomics 293:907-917
12	Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A (1994) Small
13	mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids
14	pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium
15	glutamicum. Gene 145:69-73
16	Schu DJ, Carlier AL, Jamison KP, von Bodman S, Stevens AM (2009) Structure/function
17	analysis of the Pantoea stewartii quorum-sensing regulator EsaR as an activator of
18	transcription. J Bacteriol 191:7402-7409
19	Shong J, Huang YM, Bystroff C, Collins CH (2013) Directed evolution of the
20	quorum-sensing regulator EsaR for increased signal sensitivity. ACS Chem Biol
21	8:789-795
22	Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo
23	genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nature
24	Biotechnol 1:784-791
25	Stevens AM, Queneau Y, Soulère L, von Bodman S, Doutheau A (2011) Mechanisms and
26	synthetic modulators of AHL-dependent gene regulation. Chem Rev 111:4-27

1	Studholme DJ (2011) Application of high-throughput genome sequencing to intrapathovar
2	variation in Pseudomonas syringae. Mol Plant Pathol 12:829-838
3	Taguchi F, Ogawa Y, Takeuchi K, Suzuki T, Toyoda K, Shiraishi T, Ichinose Y (2006) A
4	homologue of the 3-oxoacyl-(acyl carrier protein) synthase III gene located in the
5	glycosylation island of Pseudomonas syringae pv. tabaci regulates virulence factors via
6	N-acyl homoserine lactone and fatty acid synthesis. J Bacteriol 188:8376-8384
7	Taguchi F, Yamamoto M, Ohnishi-Kameyama M, Iwaki M, Yoshida M, Ishii T, Konishi T,
8	Ichinose Y (2010) Defects in flagellin glycosylation affect the virulence of
9	Pseudomonas syringae pv. tabaci 6605. Microbiology 156:72-80
10	Taguchi F, Inoue Y, Suzuki T, Inagaki Y, Yamamoto M, Toyoda K, Noutoshi Y, Shiraishi T,
11	Ichinose Y (2015) Characterization of quorum sensing-controlled transcriptional
12	regulator MarR and Rieske (2Fe-2S) cluster-containing protein (Orf5), which are
13	involved in resistance to environmental stresses in Pseudomonas syringae pv. tabaci
14	6605. Mol Plant Pathol 16:376-387
15	Tamura N, Murata Y, Mukaihara T (2005) Isolation of Ralstonia solanacearum hrpB
16	constitutive mutants and secretion analysis of <i>hrpB</i> -regulated gene products that share
17	homology with known type III effectors and enzymes. Microbiol. 151:2873-2884
18	Tsai CS, Winans SC (2010) LuxR-type quorum-sensing regulators that are detached from
19	common scents. Mol Microbiol 77:1072-1082
20	Tsai CS, Winans SC (2011) The quorum-hindered transcription factor YenR of Yersinia
21	enterocolitica inhibits pheromone production and promotes motility via a small
22	non-coding RNA. Mol Microbiol 80:556-571
23	Urbanowski ML1, Lostroh CP, Greenberg EP (2004) Reversible acyl-homoserine lactone
24	binding to purified Vibrio fischeri LuxR protein. J Bacteriol 186:631-637
25	von Bodman SB, Bauer WD, Coplin DL (2003) Quorum sensing in plant-pathogenic bacteria.
26	Annu Rev Phytopathol 41:455-482

1	Whitehead NA,	Barnard AM,	Slater H,	Simpson NJ,	Salmond GP (2001)	
---	---------------	-------------	-----------	-------------	-------------------	--

- 2 Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev 25:365-404
- 3
- 4

1 Figure legends

2 Fig. 1 Generation of mutant, complemented strains, and reporter plasmid. (a) Schematic

3 organization of *psyI*, *psyR*, and the surrounding region in *Pseudomonas syringae* pv. *tabaci*

4 6605 and construction of $\Delta psyR$ and its complemented strain and $\Delta psyI$ mutant. PCR was

5 carried out to isolate each DNA fragment to generate mutant or complemented strains using

6 respective primers indicated by arrows. (b) Structure of *psyI* reporter plasmid,

7 pBSLC2-*psyI-lacZYA*. Plasmid is not drawn to scale. The region between "I" and "O" is

8 randomly inserted into the bacterial genome. The *oriV* and ColE1 *ori* are replication origins

9 of R6K and colicin E1 plasmids, respectively, and *oriT* is an RP4 plasmid-derived

10 conjugative transfer origin. The *tnp* encodes transposase. Amp, ampicillin; Cm,

11 chloramphenicol; Km, kanamycin; E/H, *Eco*RV/*Hin*cII.

12 Fig. 2 AHL production. *N*-hexanoyl-L-homoserine lactone (HHL) and

13 *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) are visualized by violacein production

14 using C. violaceum CV026.

15 Fig. 3 Inoculation of host tobacco leaves by *Pta* 6605 WT and $\Delta psyR$ mutant and its

16 complemented strains by a dip method. (a) Tobacco leaves were dip-inoculated with each

17 bacterial suspension ($OD_{600} = 0.1$ in 10 mM MgSO₄) for 20 min at 23°C. Photographs taken

18 11 d after inoculation show representative results obtained from three independent

19 experiments. (b) Bacterial population was calculated at 0, 2, and 6 d post inoculation. The

20 bars represent standard deviations for three independent experiments. Asterisks indicate a

21 significant difference from the WT (*P < 0.01).

22 Fig. 4 Electrophoretic mobility shift assays (EMSA) of the rPsyR to the upstream

23 promoter-containing regions. (a) EMSA of rPsyR to the *psyI* and *psyR* promoters. The *psyI*

24 promoter (8.8 fmol) and the *psyR* promoter (4.6 fmol) were DIG-labeled, then incubated with

25 0, 280, and 480 nM of rPsyR. (b) Effect of AHL on the binding of rPsyR to the labeled *psyI*

1	promoter (8.8 fmol). Various concentrations of HHL and/or OHHL were added to the binding
2	mixture. F denotes a free DNA probe, C1 and C2 denote DNA-rPsyR complexes.
3	Fig. 5 Identification of binding site of rPsyR on <i>psyI</i> promoter. (a) DIG-labeled DNA probe
4	and series of DNA competitors. Sizes and location of DNA fragments are illustrated.
5	Numbers of the DNA fragments refer to the position of the nucleotides relative to the "A" of
6	translation start codon ATG. The dotted line indicates a mutated sequence. (b) Conserved <i>lux</i>
7	box-like sequences in the psyI promoter in Pta 6605 and Psm, luxI promoter in V. fischeri,
8	and esaR promoter in P. stewartii. The nucleotides identical to the lux like-box in Pta 6605
9	are indicated in black. The imperfect inverted repeat is indicated with arrows. (c)
10	Competition assay of binding between 480 nM of rPsyR and labeled <i>psyI</i> promoter (8.8 fmol).
11	Each non-labeled DNA (40 pmol) was added into the binding reaction mixture as competitors.
12	The reaction mixtures were separated on 10% (w/v) polyacrylamide gel.
13	Fig. 6 Promoter activity of <i>psyI</i> in <i>Pta</i> 6605. The <i>psyI</i> promoter was fused to the <i>lacZYA</i> gene,
14	and β -galactosidase activities are indicated as Miller units. (a) WT <i>Pta</i> 6605 possessing the
15	psyl promoter with a lacZYA reporter gene was incubated for 4 h to avoid the effect of
16	preculture conditions with a different cell inoculum ($OD_{600} = 0.01, 0.1, 0.5, and 1.0$). The
17	effect of bacterial cell density on β -galactosidase activity was examined. Each symbol
18	indicates the result of an independent experiment. (b) Effect of exogenous application of
19	AHL on <i>psyI</i> expression in the $\Delta psyI$ mutant. Bacterial incubation was started at OD ₆₀₀ =
20	0.015 with or without different concentrations of OHHL and HHL. The β -galactosidase
21	activity was measured after 24 h incubation. Values are expressed as the means of at least
22	three independent experiments.

Table 1 Bacterial strains and pl	lasmids used in this study
----------------------------------	----------------------------

Bacterial strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> strain		
DH5a	F^{-} , λ^{-} , $\emptyset 80dLacZ\Delta M15$, $\Delta(lacZYA-argF)U169$, recA1, endA1, hsdR17($r_{K}^{-}m_{K}^{+}$), supE44, thi-1, gvrA, relA1	Takara Bio
S17-1	Thi, pro, $hsdR^-$, $hsdM^+$, $recA$ [chr::RP4-2-Tc::Mu-Km::Tn7]	Schäfer et al. (1994)
S17-1 λpir	λpir lysogen of S17-1	Simon <i>et al.</i> (1983)
BL21 Chromobacterium violaceum CV026 P. svringae py. tabaci	<i>F</i> ⁻ , <i>ompT</i> , $hsdS_B(r_B m_B)$, <i>gal</i> , <i>dcm</i> Double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532; AHL biosensor	New England Biolabs McClean <i>et al.</i> (1997)
Isolate 6605	Wild-type, Nal ^r	Taguchi et al. (2006)
$\Delta psvR$	Isolate $6605 \Delta psvR$, Nal ^r	This study
psyR-C	Isolate 6605 $\Delta psyR$ (psyR), Nal ^r , Km ^r	This study
$\Delta psyI$	Isolate 6605 $\Delta psyI$, Nal ^r	This study
Promoter less	promoter less:: <i>lacZYA-introduced 6605</i> , Nal ^r , Km ^r	This study
WT psyl reporter	Wild-type 6605 possessing <i>psyI::lacZYA</i> , Nal ^r , Km ^r	This study
Δ <i>psyI psyI</i> reporter	Δ <i>psyI</i> possessing <i>psyI::lacZYA</i> ,, Nal ^r , Km ^r	This study
Plasmid		
pCR Blunt II TOPO	pCR Blunt II TOPO cloning vector, Km ^r	Thermo Fisher Scientific
pCR Blunt-∆ <i>psyR</i>	pCR Blunt II TOPO with 858-bp upstream and 956-bp downstream region of <i>psyR</i> , Km ^r	This study
pGEM-T Easy	Cloning vector, Amp ^r	Promega
pGEM-psyI	pGEM-T Easy possessing 2603 bp including <i>psyI</i> , Amp ^r	This study
pGEM- <i>ApsyI</i>	pGEM-T Easy possessing upstream and downstream region of <i>psyI</i> , Amp ^r	This study
pGEM-psyR	pGEM possessing 965-bp <i>psyR</i> with promoter region, Amp ^r	This study
pK18mobsacB	Small mobilizable vector, Km ^r , sucrose-sensitive (<i>sacB</i>)	Schäfer et al. (1994)
pK18-∆ <i>psyR</i>	pK18mobsacB with 858-bp upstream and 956-bp downstream region of <i>psyR</i> , Km ^r	This study
pK18-Δ <i>psyI</i>	pK18mobsacB with <i>psyI</i> -deleted DNA fragment, Km ^r	This study
pBSL118	Mini-Tn5 derived plasmid vector for insertion	Alexeyev <i>et al.</i> (1995)
pBSL-psyR	pBSL118 possessing 965-bp <i>psyR</i> with promoter region. Amp ^r , Km ^r	This study
pMAL-c5X-His	Protein expression vector with maltose-binding protein, Amp ^r	New England Biolabs
pMAL-psyR	pMAL-c5X-His with 741 bp of <i>psyR</i> open reading frame	This study
pHSG396	General purpose plasmid vector, Cm ^r	Takara Bio
pBSLC2	Transposon vector constructed by ligation of <i>Eco</i> RV-digested pBSL118 and <i>Hin</i> cII-digested pHSG396, Amp ^r , Km ^r , Cm ^r	This study

pARO-lacZYA	pARO191 possessing <i>lacZYA</i>	Tamura et al. 2005
pBSLC2-lacZYA	pBSLC2 possessing <i>lacZYA</i>	This study
pBSLC2-psyI-lacZYA	pBSLC2-lacZYA possessing psyI promoter	This study

Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance, Km^r, kanamycin resistance, Nal^r, nalidixic acid resistance

Oligonucleotide	Sequence (5'-3')	Purpose	
name			
psyR-5F	AGCCTGCGTCTCGCTTAA	PCR amplification of upstream	
psyR-5R	gggatccTGGCTGTACTCATTATCCGC	region of <i>psyR</i> for cloning	
psyR-3F	cgggatccTGACTCAGGCAGCGCTGTG	PCR amplification of downstream	
psyR-3R	CAGGAACCGCGTTCAAGGTG	region of <i>psyR</i> for cloning	
psyI-1	TGGTGTCTTGTAGCGGCCAG	PCR amplification of <i>psyI</i> and	
psyI-2	AATCCCATCCGGTGTGCGTG	surrounding region for cloning	
psyI-3	gctctagaGAAACTCAAACCCGCTCGACAT	Inverse PCR to delete psyl coding	
psyI-4	gctctagaCTGAGTCAGACCATGCCCAT	region to generate $\Delta psyI$	
psyR-1	ACAAAATCCCATCCGGTGTG	PCR amplification of <i>psyR</i> with	
psyR-2	TCA GACCATGCCCATGTTGAT	promoter region for complementation	
psyR-F	ccat <u>atg</u> GAGGTTCGTACCGTGAAAGC	PCR amplification to introduce <i>psyR</i>	
psyR-R	ggaattcGACCATGCCCATGTTGATGGC	into pMALc5X-His	
psyI-PF	AGAACGCCGAAGCAGATTTC	PCR to prepare <i>psyl</i> DNA probe (153	
psyI-PR	CCTTGCACAACCAGTGTGAA	bp) for EMSA	
psyR-PF	CACTACTCCTTGCTGTCGGA	PCR to prepare psyR DNA probe	
psyR-PR	GTAATACCTTGTTTATTGTTTTGTTCTCGG	(290 bp) for EMSA	
60-S	TGTTATATTTCAAGGTGTTGACCTGTTCTTAGGTA	DNA probe for EMSA	
	CAGTAGATTTACCGTTTGAAATACG		
60-AS	CGTATTTCAAACGGTAAATCTACTGTACCTAAGA		
	ACAGGTCAACACCTTGAAATATAACA		
60m-S	TGTTATATTTCAAGGTGTTGacgtacgtacgtacgtacgtGAT	DNA probe for EMSA	
	TTACCGTTTGAAATACG		
60m-AS	CGTATTTCAAACGGTAAATCacgtacgtacgtacgtacgtA		
	ACACCTTGAAATATAACA		
5'-20-S	TGTTATATTTCAAGGTGTTG	DNA probe for EMSA	
5'-20-AS	CAACACCTTGAAATATAACA		
Core-S	ACCTGTTCTTAGGTACAGTA	DNA probe for EMSA	
Core-AS	TACTGTACCTAAGAACAGGT		
3'-20-S	GATTTACCGTTTGAAATACG	DNA probe for EMSA	
3'-20-AS	CGTATTTCAAACGGTAAATC		
psyI-F	ggactagtACCATTATCCCTATGGAGTCAT	PCR to prepare <i>psyI</i> promoter for	
psyI-R	cgggatccTACGTAACGGGCATCGTCGTG	construction of reporter plasmid	

Table 2 Oligonucleotide sequences used in this study.

Underlined letters indicate the translation start codon in psyR-F and the antisense for translation stop codon of *psyR* in psyR-2. Small letters indicate "additive nucleotides" containing artificial *Bam*HI sites in psyR-5R, psyR-3F and psyI-R, *Nde*I site in psyR-F, *Eco*RI site in psyR-R, *Xba*I sites in psyI-3 and psyI-4 and *Spe*I site in psyI-F, respectively. Small letters in 60m-S and 60m-AS indicate mutated sequence.



Fig. 1. Generation of mutant, complemented strains, and reporter plasmid. (a) Schematic organization of *psyI*, *psyR*, and the surrounding region in *Pseudomonas syringae* pv. *tabaci* 6605 and construction of $\Delta psyR$ and its complemented strain and $\Delta psyI$ mutant. PCR was carried out to isolate each DNA fragment to generate mutant or complemented strains using respective primers indicated by arrows. (b) Structure of *psyI* reporter plasmid (pBSLC2-*psyI-lacZYA*). Plasmid is not drawn to scale. The region between "I" and "O" is randomly inserted into the bacterial genome. The *oriV* and ColE1 *ori* are replication origins of R6K and colicin E1 plasmids, respectively, and *oriT* is an RP4 plasmid-derived conjugative transfer origin. The *tnp* encodes transposase. Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; E/H, *Eco*RI/*Hin*cII.



Fig. 2. AHL production. *N*-hexanoyl-L-homoserine lactone (HHL) and *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) are visualized by violacein production using *C. violaceum* CV026.



Fig. 3. Inoculation of host tobacco leaves by *Pta* 6605 WT and $\Delta psyR$ mutant and its complemented strains by a dip method. (a) Tobacco leaves were dip-inoculated with each bacterial suspension ($OD_{600} = 0.1$ in 10 mM MgSO₄) for 20 min at 23°C. Photographs taken 11 d after inoculation show representative results obtained from three independent experiments. (b) Bacterial population was calculated at 0, 2, and 6 d post inoculation. The bars represent standard deviations for three independent experiments. Asterisks indicate a significant difference from the WT (**P*<0.01).



Fig. 4. Electrophoretic mobility shift assays (EMSA) of the rPsyR to the upstream promotercontaining regions. (a) EMSA of rPsyR to the *psyI* and *psyR* promoters. The *psyI* promoter (8.8 fmol) and the *psyR* promoter (4.6 fmol) were DIG-labeled, then incubated with 0, 280, and 480 nM of rPsyR. (b) Effect of AHL on the binding of rPsyR to the labeled *psyI* promoter (8.8 fmol). Various concentrations of HHL and/or OHHL were added to the binding mixture. F denotes a free DNA probe, C1 and C2 denote DNA-rPsyR complexes.



Fig. 5. Identification of binding site of rPsyR on *psyI* promoter. (a) DIG-labeled DNA probe and series of DNA competitors. Sizes and location of DNA fragments are illustrated. Numbers of the DNA fragments refer to the position of the nucleotides relative to the "A" of translation start codon ATG. The dotted line indicates a mutated sequence. (b) Conserved *lux* box-like sequences in the *psyI* promoter in *Pta* 6605 and *Psm, luxI* promoter in *V. fischeri*, and *esaR* promoter in *P. stewartii*. The nucleotides identical to the *lux* like-box in *Pta* 6605 are indicated in black. The imperfect inverted repeat is indicated with arrows. (c) Competition assay of binding between 480 nM of rPsyR and labeled *psyI* promoter (8.8 fmol). Each non-labeled DNA (40 pmol) was added into the binding reaction mixture as competitors. The reaction mixtures were separated on 10% (w/v) polyacrylamide gel.



Fig. 6. Promoter activity of *psyI* in *Pta*6605. The *psyI* promoter was fused to the *lacZYA* gene, and β -galactosidase activities are indicated as Miller units. (a) Wild-type *Pta*6605 possessing the *psyI* promoter with a *lacZYA* reporter gene was incubated for 4 h to avoid the effect of preculture conditions with a different cell inoculum (OD₆₀₀ = 0.01, 0.1, 0.5, and 1.0). The effect of bacterial cell density on β -galactosidase activity was examined. Each symbol indicates the result of an independent experiment. (b) Effect of exogenous application of AHL on *psyI* expression in the $\Delta psyI$ mutant. Bacterial incubation was started at OD₆₀₀ = 0.015 with or without different concentrations of OHHL and HHL. The β -galactosidase activity was measured after 24 h incubation. Values are expressed as the means of at least three independent experiments.