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# Vfr targets promoter of genes encoding methyl-accepting chemotaxis protein in *Pseudomonas syringae* pv. *tabaci* 6605

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ABSTRACT

Virulence factor regulator (Vfr) is an indispensable transcription factor in the expression of virulence in the phytopathogenic bacteria *Pseudomonas syringae*. However, the function of Vfr is not known so far. The deletion of *vfr* resulted in the loss of surface swarming motility and reduced the virulence in *P. syringae* pv. *tabaci* (*Pta*) 6605. In order to identify the target genes of Vfr, we screened the sequences that bind to Vfr by chromatin immune precipitation (ChIP) and sequencing methods using the closely related bacterium *P. syringae* pv. *syringae* (*Pss*) B728a. For this purpose we first generated a strain that possesses the recombinant gene *vfr*::*FLAG* in *Pss* B728a, and performed ChIP using an anti-FLAG antibody. Immunoprecipitated DNA was purified and sequenced with Illumina HiSeq. The Vfr::FLAG-specific peaks were further subjected to an electrophoresis mobility-shift assay, and the promoter regions of locus tag for Psyr_0578 , Psyr_1776, and Psyr_2237 were identified as putative target genes of Vfr. These genes encode plant pathogen–specific methyl-accepting chemotaxis proteins (Mcp). These *mcp* genes seem to be involved in the Vfr-regulated expression of virulence.

Virulence factor regulator (Vfr) is known to be a member of the cyclic 3′,5′adenosine monophosphate (cAMP) receptor proteins, and is essential for the synthesis of cAMP. The function of Vfr is relatively well studied in a human opportunistic pathogenic bacterium, *Pseudomonas aeruginosa* [1,2]. Vfr binds to the promoter of many virulence-associated genes such as *algD*, *pbpG*, *plcN*, *plcHR*, *prpL*, *vfr*, *pilM/ponA*, *regA*, and *toxA*, and these promoters contain a putative Vfr-binding sequence, 5′-ANWWTGNGAWNYAGWTCACAT-3’ [3]. Because Vfr binds its own promoter, transcription of *vfr* is self-regulated. Vfr autoregulates *vfr* expression through a cAMP-dependent mechanism [4]. It is also known that Vfr directly activates *exsA* transcription. Because, the ExsA is the central regulator of T3SS gene expression, Vfr regulates T3SS gene expression by controlling the expression of ExsA [2]. Recently, it was revealed that Vfr also binds and controls expression of the *exlBA* promoter [1]. ExlBA constitutes the two-partner secretion system involved in a main virulence determinant of *P. aeruginosa*, exolysin [1].

In plant-associated bacteria, Vfr is an important virulence regulator required for the expression of flagella-, pili-, and T3SS-related genes in *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta*6605) [5]. To our knowledge, this is the only report of Vfr in plant pathogenic bacteria. In other plant-associated bacteria, Vfr is known to be involved in the production of antibiotics to control fungal pathogens produced by biocontrol bacteria such as *Pseudomonas fluorescens* FD6 [6] and *Pseudomonas chlororaphis* G05 [7]. The *vfr* mutant of *P. fluorescens* FD6 enhanced the production of the antibiotics 2,4-diacetylphloroglucinol, pyrrolnitrin, and pyoluteorin, biofilm production, swimming motility, and expression of exopolysaccharide-related genes (*pelA*, *pslA*, and *pslB*), but reduced protease production [6]. Analysis of the *vfr* mutant of *P. chlororaphis* G05 showed that Vfr is required for pyrrolnitrin production but not for phenazine-1-carboxylic acid biosynthesis [7]. These results indicate that Vfr functions differently in *Pta*6605 compared to biocontrol bacteria.

Here, we focused on identifying the targeted genes of Vfr in *P. syringae* pv. *syringae* B728A (*Pss*B728a). *Pss*B728a is one of model phytopathogenic bacterium of *P. syringae,* and whole genomic sequence was determined [8]. All bacterial strains and plasmids used in this study were listed in Table S1. Vfr is a well-conserved protein and its amino acid sequence shows 96–100% identities among *P. syringae* strains. To identify the targeted genes of Vfr, candidate DNA was isolated by chromatin immunoprecipitation and sequencing (ChIP-seq) using *Pss*B728a. *Pss*B728a was maintained in King’s B (KB) medium at 27°C.

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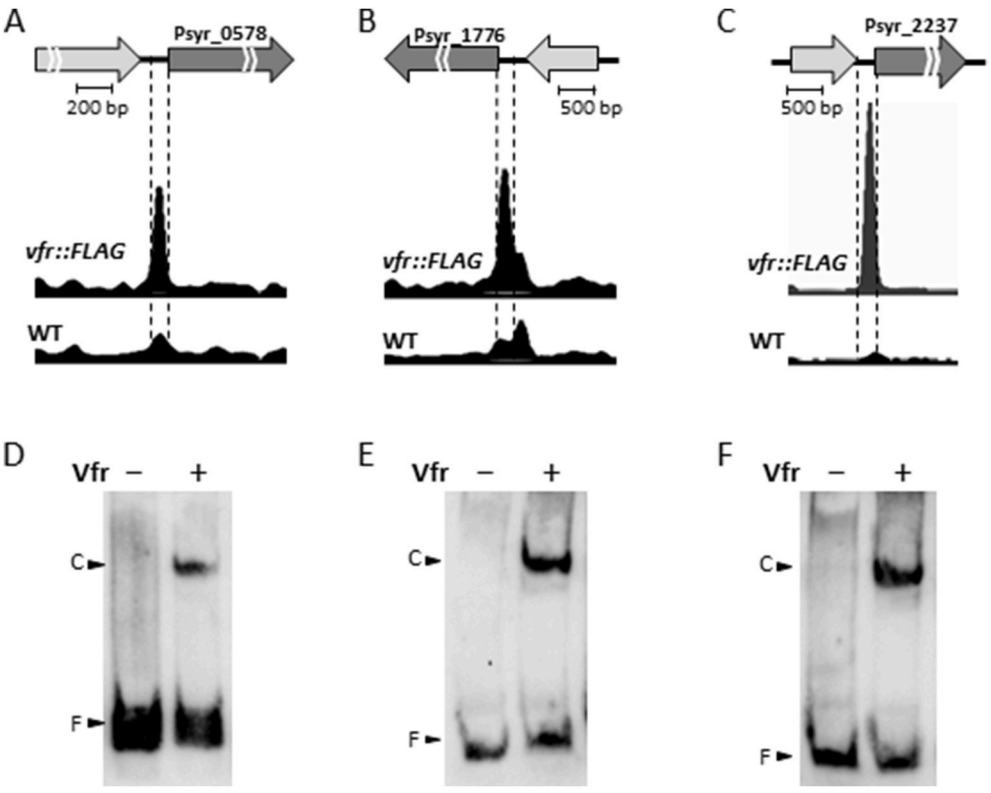


**Fig. 1.** Visualization of the sequencing results obtained by chromatin immunoprecipitation (A–C) and electrophoretic mobility shift assays (EMSA) of the rVfr to the upstream promoter region of obtained candidate genes as target gene (D–F). The numbers of the reads were counted for each sample (*vfr*::*FLAG* and WT), and are represented as profiles. Dotted areas are the region of DIG probe used in EMSA in D-F. The arrows indicate the direction of the open reading frames of genes, and dark shadowed genes (A: Psyr_0578, B: Psyr_1776, and C: Psyr_2237) are thought to be candidates for the Vfr target gene. EMSA of rVfr to the promoters of Psyr_0578 (D), Psyr_1776 (E), and Psyr_2237 (F) are shown. The reaction mixtures were separated on 10% (W/V) polyacrylamide gel. Each DIG-labeled promoter fragment (0.8 ng) was incubated with 0 or 310 ng of rVfr. F and C denote a free DNA probe and a DNA-rVfr complex, respectively.

First, we replaced *vfr* with *vfr*::*FLAG* in *Pss*B728a to use anti-FLAG antibody (Merck, Darmstadt, Germany) for ChIP. Using genomic DNA of *Pss*B728a as a template, PCR was carried out with the sets of primers listed in Table S2 to add FLAG tag peptide (DYKDDDDK) at the C-terminus of Vfr. PCR products of the open reading frame of *vfr* with its upstream region and the downstream region of *vfr* were mixed after removing the primers, then PCR was performed again without addition of primers. The resultant PCR product was inserted into the small mobilizable vector pK18*mobsacB* [9] to obtain pK18*mobsacB-vfr*::*FLAG*. This plasmid was introduced into *E. coli* S17-1, and *vfr* was replaced with *vfr*::*FLAG* in *Pss*B728a by conjugation and homologous recombination according to the method described previously [10]. Because the swarming motility was lost in the Δ*vfr* mutant of *Pta*6605 [5], we investigated whether the swarming motility of a strain B728a *vfr*::*FLAG* was retained. As shown in Fig. S1, *Pss*B728a *vfr*::*FLAG* still showed swarming ability, suggesting that the Vfr-FLAG was functional, and available for use in ChIP-seq experiment, although the swarming motility was reduced comparing to that of the WT.

ChIP-seq analysis was performed by the previously described method [11] with slight modifications. WT and *vfr*::*FLAG* of *Pss*B728a were incubated overnight at 27°C in KB medium, then further incubated for 1 h in fresh MMMF medium [5]. Then, bacteria were treated with 1.2% formaldehyde at final concentration with gentle shaking for 90 min. Cross-linking was quenched by the addition of glycine to 0.33 M final concentration for 5 min. The cells were harvested and sonicated to obtain genomic DNA fragments of smaller than 500 bp. The resultant DNA was precipitated with an anti-FLAG M2 affinity gel (Merck), and DNA containing Vfr-binding sequences was purified using a Spin-X centrifuge tube filter (Sigma-Aldrich, St. Louis, MO, USA). The quality and quantity of the obtained DNA was confirmed by a Bioanlyzer 2100 (Agilent, Santa Clara, CA, USA) and sequenced by HiSeq (Illumina, San Diego, CA, USA). Reads were aligned to the *Pss*B728a genome (NCBI accession numbers: NC_007005.1) using an ultrafast, memory-efficient short read aligner, Bowtie (version 1.0.0) (http://bowtie-bio.sourceforge.net/index.shtml). The numbers of raw Illumina reads in ChIP-seq analyses of WT and *vfr*::*FLAG* are $2.00 \times 10^7$ and $2.17 \times 10^7$, respectively. Obtained sequences were aligned to the *Pss*B728a genome, DNA enrichments by ChIP were visualized as specific peaks containing large numbers of sequences in the *vfr*::*FLAG* strain, and 20 DNA fragments were detected as candidate of Vfr-targeted genes (Table S3). The promoter regions of these candidate genes were PCR-amplified, DIG-labeled according to the DIG Gel Shift Kit 2nd Generation (Sigma-Aldrich) and subjected to the electrophoretic mobility shift assay (EMSA) using recombinant Vfr protein (rVfr) as previously described [10]. To produce rVfr PCR-amplified *vfr* sequence was inserted into the pMALc5X-His (New England Biolabs, Ipswich, MA, USA), and rVfr was purified according to the manufacturer's instructions as a fusion protein with maltose-binding protein (MBP). The rVfr was further purified using a column of amylose resin (New England Biolabs).

To examine the potential binding of the Vfr and targeted genes an EMSA was employed. Among 20 candidate genes, we observed three positive interactions between rVfr and promoters of Psyr_0578, Psyr_1176, and Psyr_2237. We observed specific peaks in each promoter region of vfr::FLAG strain but not of WT (Fig. 1A–C). A clear shift of the band corresponding to each promoter was observed by the addition of rVfr (Fig. 1D–F). Genes of Psyr_0578, Psyr_1776, and Psyr_2237 encode methyl-accepting chemotaxis proteins (MCPs). These results indicate that Vfr binds to promoter region of each *mcp* gene. We also confirmed putative Vfr-binding site in the promoter of each *mcp* gene (Fig. S2A). Some promoters possess putative Vfr-binding sites, although we did not observe band shift in EMSA (Fig. S2B).

MCP is chemoreceptor proteins, whose function in the cell includes the control of the direction of movement according to the presence of attractants or repellents. Upon binding such chemotactic ligands to MCP, chemotaxis signals are generated and transmitted to flagellar motors via a set of chemotaxis proteins [12]. Furthermore, it is known that phytopathogenic bacteria possess a relatively large number of *mcp* genes. For example, *P. syringae* pv. *tomato* DC3000 (*Pto*DC3000) genome encodes 49 *mcp* genes, whereas *P. aeruginosa* PAO1 has 25 *mcp* genes [13], indicating that phytopathogenic bacteria have many MCPs

including chemoreceptors for plant-derived molecules.

Vfr is an indispensable transcription factor for virulence in *Pta*6605 [5]. The Δ*vfr* mutant had reduced virulence, swimming and swarming motilities, defense response in nonhost *Arabidopsis*, and concentration of cAMP. Phenotypic changes of the Δ*vfr* mutant were accompanied by the changes in the gene expression profile. The microarray and qRT-PCR analyses of WT and Δ*vfr* mutant strains revealed that the *vfr* mutant showed reduced expression of genes for the T3SS, type IV pilus biogenesis, flagellum biogenesis, iron uptake, and biosynthesis of second messenger cAMP [5]. Therefore, Vfr seems to regulate the expression of various targeted genes. However, we identified only three *mcp* genes as target of Vfr by ChIP-seq and EMSA screening. We had expected that Vfr controls not only the three *mcp* genes, but also other virulence related genes. Probably our screening was too strict or the addition of FLAG to Vfr may have affected the binding to the targeted DNA.

Three *mcp* genes might be involved in the Vfr-regulated expression of virulence. It is known that chemotaxis is important for bacterial virulence. Gathering of *Pto*DC3000 around open stomata of *Arabidopsis thaliana* leaves suggested that *Pto*DC3000 can sense chemical signals released from the stomata [14]. Actually, the mutation of *cheA2* in *Pto*DC3000 eliminated surface motility and reduced virulence on tomato and *Arabidopsis* [15]. We also found the requirements of *cheA2* and *cheY2* of *Pta*6605 in chemotaxis and virulence on host tobacco plant (unpublished results). Recently, it was reported that the defective mutant of chemoreceptor of amino acid, PscA in *Pto*DC3000 and chemoreceptor of γ-aminobutyric acid, McpG in *Pta*6605 reduced virulence [16,17]. Furthermore, PscA and McpG are not only chemoreceptors but also regulator of virulence related traits. Thus, it seems common that bacterial chemotaxis is important for virulence. What are the ligands for the MCP encoded by Psyr_0578, Psyr_1776, and Psyr_2237? Based on the number of transmembrane domains, the presence or absence of ligand binding domain and its localization, MCPs can be classified into seven topology types (Ia, Ib, II, IIIm, IIIc, IVa, and IVb) [18]. All Psyr_0578, Psyr_1776, and Psyr_2237 encodes type Ia MCP. However, their ligands are not known yet. Further investigation is necessary to reveal the function of these MCPs and whole mechanism of Vfr-mediated expression of virulence in *Pta*6605.

## Author statement

Keisuke Ogura: Preparation and experiments, Hidenori Matsui: Data curation, Mikihiro Yamamoto: Software, Visualization, Yoshiteru Noutoshi: Discussion, Kazuhiro Toyoda: Discussion, Fumiko Taguchi: Methodology and Experiments, Yuki Ichinose: Project administration and writing, Funding acquisition.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100944.

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