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 swimming 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 immunoprecipitation (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 Pssyr_0578, Pssyr_1776, and Pssyr_2237 were identified as putative target genes of Vfr. These genes encode plant pathogen-specific methyl-accepting chemotaxis proteins (Mcp). These mcps 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, pchG, plcN, plcHR, prpL, vfr, pilM/pomA, regA*, and *toxA*, and these promoters contain a putative Vfr-binding sequence, 5’-ANWWTTNGAWNYAGTCACAT-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. However, 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 the plant-associated bacteria, *Pss* 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-diacylphloroglucinol, pyrrolnitrin, and pyoluteorin, biofilm production, swimming motility, and expression of exopolysaccharide-related genes (*pelA, pilA*, 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 (*PssB728a*). *PssB728a* is one of model phytopathogenic bacterium of *Prs*, 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 *Prs* strains. To identify the targeted genes of Vfr, candidate DNA was isolated by chromatin immunoprecipitation and sequencing (ChIP-seq) using *PssB728a*. *PssB728a* was maintained in King’s B (KB) medium at 27°C.
First, we replaced vfr with vfr::FLAG in PssB728a to use anti-FLAG antibody (Merck, Darmstadt, Germany) for ChIP. Using genomic DNA of PssB728a 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 pK18mobsacB [9] to obtain pK18mobsacB-vfr::FLAG. This plasmid was introduced into E. coli S17-1, and vfr was replaced with vfr::FLAG in PssB728a by conjugation and homologous recombination according to the method described previously [10]. Because the swelling motility was lost in the ∆vfr mutant of Pta6605 [5], we investigated whether the swelling motility of a strain B728a vfr::vfr was still showed swelling ability, suggesting that the Vfr-FLAG was functional, and available for use in ChIP-seq experiment, although the swelling 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 PssB728a 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 Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and sequenced by HiSeq (Illumina, San Diego, CA, USA). Reads were aligned to the PssB728a 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 × 10^7 and 2.17 × 10^7, respectively. Obtained sequences were aligned to the PssB728a 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_1176, 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 (PtoDC3000) 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 \textit{Pta}6605 [5]. The \(\Delta vfr\) mutant had reduced virulence, swimming and swimming motilities, defense response in nonhost \textit{Arabidopsis}, and concentration of cAMP. Phenotypic changes of the \(\Delta vfr\) mutant were accompanied by the changes in the gene expression profile. The microarray and qRT-PCR analyses of WT and \(\Delta vfr\) 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 \textit{mcp} genes as target of Vfr by ChiP-seq and EMSA screening. We had expected that Vfr controls not only the three \textit{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 \textit{mcp} genes might be involved in the Vfr-regulated expression of virulence. It is known that chemotaxis is important for bacterial virulence. Gathering of \textit{PtoDC3000} around open stomata of \textit{Arabidopsis thaliana} leaves suggested that \textit{PtoDC3000} can sense chemical signals released from the stomata [14]. Actually, the mutation of \textit{cheA2} in \textit{PtoDC3000} eliminated surface motility and reduced virulence on tomato and \textit{Arabidopsis} [15]. We also found the requirements of \textit{cheA2} and \textit{cheY2} of \textit{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 \textit{PtoDC3000} and chemoreceptor of \(\gamma\)-amino butyric acid, McpG in \textit{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 \textit{PsyR}, \textit{PsyR}, and \textit{PsyR}, 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, IIC, Iva, and Ivb) [18]. All \textit{PsyR}, \textit{PsyR}, and \textit{PsyR}, encode 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 \textit{Pta}6605.

Author statement

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

Declaration of competing interest

None.

Acknowledgments

We would like to thank the Leaf Tobacco Research Laboratory of Japan Tobacco Inc. for providing \textit{Pta} 6605. This work was supported in part by Grants-in-Aid for Scientific Research (nos. 16K14681 and 19H02956) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

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