Phytotoxin synthesis genes and type III effector genes of *Pseudomonas syringae* pv. *actinidiae* biovar 6 are regulated by culture conditions

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Abstract

The kiwifruit bacterial canker (*Pseudomonas syringae* pv. *actinidiae*; Psa) causes severe damage to kiwifruit production worldwide. Psa biovar 6 (Psa6), which was isolated in Japan in 2015, produces two types of phytotoxins: coronatine and phaseolotoxin. To elucidate the unique virulence of Psa6, we performed transcriptomic analysis of phytotoxin synthesis genes and type III effector genes in *in vitro* cultivation using various media. The genes related to phytotoxin synthesis and effectors of Psa6 were strictly regulated in the coronatine-inducing mediums (HS and HSC); 14 of 23 effector genes and a *hrpL* sigma factor gene were induced at 3 h after transferring to the media (early-inducible genes), and phytotoxin synthesis genes such as *argD* of phaseolotoxin and *cfl* of coronatine were induced at 6 and 12 h after transferring to the media (late-inducible genes). In contrast, induction of these genes was not observed in the *hrp*-inducing medium. Next, to examine whether the changes in gene expression in different media is specific to Psa6, we investigated gene expression in other related bacteria. For Psa biovar 1 (Psa1), biovar 3 (Psa3), and *P. s.* pv. *glycinea* (Psg), no clear trends were observed in expression behavior across various culture media and incubation times. Therefore, Psa6 seems to exert its virulence efficiently by using two phytotoxins and effectors according to environmental changes. This is not seen in other biovars and pathovars, so it is thought that Psa6 has acquired its own balance of virulence.

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Introduction

41

42 Kiwifruit belong to the family *Actinidiaceae* and the genus *Actinidia*. They include *Actinidia*

43 *deliciosa*, a species whose flesh is green, and *A. chinensis*, a species whose flesh is yellow or red.

44 In Japan, kiwifruit seedlings were first introduced from New Zealand in the 1970s and are now

45 popular as edible fruit (*Ushiyama, 1993*). However, around 1980, kiwifruit bacterial canker

46 broke out and caused serious damage to kiwifruit production (*Takikawa et al., 1989*). Since then,

47 this disease has resulted in economic and productive damage in kiwifruit orchards throughout

48 Japan.

49 The pathogen that causes kiwifruit bacterial canker is *Pseudomonas syringae* pv.

50 *actinidiae* (Psa). It has been further classified into biovar 1, 2, 3, 5, and 6, reflecting its

51 physiological and genetic diversity (*Chapman, et al., 2012*; *Fujikawa & Sawada, 2016*; *2019*;

52 *Sawada & Fujikawa, 2019a*; *2019b*). In Japan, Psa is highly diverse, with significant differences

53 in the types or presence of phytotoxins produced among biovars (*Sawada & Fujikawa, 2019a*;

54 *2019b*). In general, the plant pathogenic bacteria group, *P. syringae,* is highly differentiated in

55 pathogenicity, and it is known to have more than 60 different pathovars with different host plants

56 and symptoms (*Sawada, 2014*). However, of all known *P. syringae* pathogens, only Psa biovar 6

57 (Psa6) has been reported to produce two phytotoxins (coronatine and phaseolotoxin)

58 simultaneously. Phytotoxins are known to be important factors in the virulence of plant

59 pathogens because they have a significant effect on plants, even at low concentrations (*Bender,*

60 *1999*; *Strange, 2007*). Coronatine, a phytotoxin produced by Psa6, is structurally similar to the

61 plant hormone jasmonic acid (*Geng et al., 2014*). It has been reported to inhibit host defense

62 responses by disrupting hormonal signals and reopening closed stomata (*Geng et al., 2014*).

63 Phaseolotoxin, another phytotoxin produced by Psa6, has been shown to induce halos around

64 lesions in leaves by inhibiting the function of ornithine carbamoyltransferase, which is an

65 enzyme involved in arginine biosynthesis in host plants (*Mitchell, 1976*; *Patil et al., 1976*;

66 *Tamura et al., 2002*). These phytotoxins have been extensively studied, and the conditions under

67 which they are induced have been clarified in some bacteria. For example, *P. syringae* pv.

68 *glycinea* (Psg), which is a causal pathogen of soybean (*Glycine max*), produces coronatine when

69 cultured in HS and HSC medium (*Hoitink & Sinden, 1970*; *Palmer & Bender, 1993*), and *P.*

70 *syringae* pv. *phaseolicola*, which is a causal pathogen of the common bean (*Phaseolus vulgaris*),

71 produces the highest amounts of phaseolotoxin at 18 ℃ (*Nüske & Fritsche, 1989*).

72 In addition to the two phytotoxins, Psa6 produces other virulence factors, type III effector

73 proteins. Effectors are known to suppress or interfere with the plant's defense response,

74 increasing the pathogen's virulence (*Jones & Dangl, 2006*). The type III secretion system is a

75 specialized molecular machine of gram-negative bacteria that allows the effector proteins to be

76 injected directly into the plant host cells (*Coombes, 2009*). Effector genes of Psa6 in the early

77 stages of infection can be classified into several groups based on differences in expression

78 patterns (*Fujikawa & Sawada, 2019*). Although the core genome sequences of Psa are almost the

79 same among biovars, the presence and combination of phytotoxins and effector genes are
80 diverse. Therefore, the diversity of effectors and phytotoxins may be due to accessory genomes
81 encoded by transposons and other mobile genetic elements (*Sawada & Fujikawa, 2019a*; *2019b*).
82   It is important to understand the survival strategies, evolution, and natural adaptations of
83 Psa. In particular, determining the expression behavior of the two phytotoxin synthesis genes and
84 effector genes of Psa6 can provide important information. We used RNA sequencing (RNA-seq)
85 and reverse transcription-quantitative PCR (RT-qPCR), to perform transcriptomic analysis of
86 these genes when cultured under phytotoxin-inducing or effector-inducing conditions.
87
88

89 ## Materials & Methods
90 **Bacterial strains, media, and culture conditions**
91 Psa6 MAFF 212134, Psa1 MAFF 302145, Psa3 MAFF 212104, and Psg MAFF 301683 were
92 used as the representative strains. These strains can be obtained from NARO Genebank
93 (https://www.gene.affrc.go.jp/index_en.php). For the pre-culture, each strain was scraped from
94 LB (*Bertani, 1951*) solid medium and was transferred to new LB liquid media. The cultures were
95 then shaken overnight. After shaking, the pre-cultures were centrifuged at $6,000 \times g$ for 5 m, then
96 each pellet was resuspended with water to OD (660 nm) = 0.6. Two milliliters of resuspension
97 were inoculated into 15 mL of respective test media and the cultures were shaken at 160 rpm at
98 27 or 18 ºC. As test media, LB medium, HS medium, HSC medium, and *hrp*-inducing medium
99 were used (Table 1). HS and HSC media have been reported to activate coronatine production of
100 Psg (*Hoitink & Sinden, 1970*; *Palmer & Bender, 1993*). On the other hand, *hrp*-inducing medium
101 has been reported to induce the expression of *hrp* genes and effector genes of Psg (*Huynh et al.,*
102 *1989*). Additionally, it has been reported that cultures at 18 ºC, compared to 27 ºC, induce the
103 production of phytotoxins such as coronatine and phaseolotoxin (*Nüske & Fritsche, 1989*;
104 *Palmer & Bender, 1993*). After incubation at 0, 3, 6, and 12 h, 1 mL of each culture was taken
105 and used for extraction of DNA or RNA.
106

107 **DNA and RNA extraction**
108 DNA was extracted using the DNeasy mini kit (QIAGEN, Hilden, Germany) and RNA was
109 extracted using the RNeasy mini kit (QIAGEN). DNA was used to confirm primer design, and
110 RNA was used for RNA-Seq and RT-qPCR.
111

112 **RNA-Seq**
113 RNA-Seq was performed according to our previous study (*Fujikawa & Sawada, 2019*). Briefly,
114 total RNA was extracted from the 3 h culture in HS or HSC media at 27 ºC or 18 ºC, were treated
115 with the RiboMinus Transcriptome Isolation Kit for bacteria (Thermo Fisher Scientific Inc.,
116 Waltham, MA, USA), and rRNAs were almost depleted from the total RNAs. The RNA-Seq
117 library was constructed from sample RNAs using the Ion Total RNA-Seq Kit v2 (Thermo Fisher
118 Scientific Inc.) and Ion Xpress RNA-Seq barcode (Thermo Fisher Scientific Inc.). Subsequently,

119   the RNA-Seq templates were prepared using the Ion PGM Hi-Q View OT2 Kit (Thermo Fisher
120   Scientific Inc.) on an Ion OneTouch 2 system. The templates were sequenced with an Ion PGM
121   Hi-Q View Sequencing Kit (Thermo Fisher Scientific Inc.) and a 318 Chip Kit v2 (Thermo
122   Fisher Scientific Inc.) on an Ion PGM next generation sequencer. Sequence data were analyzed
123   as RNA reads using the CLC Genomics Workbench ver. 11 (QIAGEN) and the statistical
124   software *R* ver. 3.5.1 with various packages (*edgeR*, *limma*, and *gplots*) according to a previous
125   report (*Fujikawa & Sawada, 2019*). Namely, using high-quality sequence reads with the
126   reference genome of Psa6 MAFF 212134 (accession no. MSBW00000000), the reads per
127   kilobase of exon per million mapped reads (RPKM) values were obtained and normalized. Then,
128   using *R* software, the gene expression values relative to the 27 ºC HS medium were calculated to
129   $\log_2$ values of fold change ($\log_2$FC) with FDR (false discovery rate) values and each effect size
130   (eta squared) values under the four culture conditions.
131   For the RT-qPCR analysis, reference gene candidates were selected according to the following
132   conditions: (1) low FC (fold change) value, which means that expression is not strongly affected
133   by culture conditions ($\log_2$FC= $\leq \pm$ 0.2), and (2) high and stable CPM (counts per million) value
134   (sum of $\log_2$CPM). When genes meeting the above conditions were found, primers were
135   designed and used for RT-qPCR analysis.
136
137   **RT-qPCR**
138   For RT-qPCR analysis, cDNA was synthesized from total RNA of each culture sample using the
139   PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) with random hexamers
140   to yield a total volume of 20 µL. Then, 180 µL of nuclease-free water was added to bring the
141   total volume to 200 µL. The synthesized cDNA was subjected to RT-qPCR using TB Green
142   Premix Ex Taq II (Takara Bio). The PCR conditions were set as follows: denaturation at 96 ºC
143   for 10 m; a cycle of 96 ºC for 15 s, 58 ºC for 30 s, and 72 ºC for 1 m, repeated 40 times; then 96
144   ºC for 15 s, 58 ºC for 1 m, and 95 ºC for 15 s to form a melting curve. DNA of Psa6 was used as
145   a positive control and nuclease-free water was used as a negative control. Then, based on the
146   results of RNA-Seq (described above) we selected the genes that had similar expression levels
147   across all culture conditions as reference genes for each bacterium. According to the method of
148   *Smith et al.* (*2018*), RT-qPCR results were processed using the following equation:

149
$$NRQ = \frac{E_{GOI}^{-(Ct_{GOI\ in\ sample} - Ct_{GOI\ in\ 0h\ sample})}}{\sqrt[n]{\prod_{k=1}^{n} E_{RG}^{-(Ct_{RG\ in\ sample} - Ct_{RG\ in\ 0h\ sample})}}}$$

150   …..(1)
151   Where NRQ is the normalized relative quantity, *E* is amplification efficiency of qPCR, GOI is
152   gene of interest, and RG is reference gene
153   To solve the NRQ (*ΔΔ*Ct value), a geometric mean was calculated from the RT-qPCR results
154   of three reference genes as shown by the denominator equation, and *Δ*Ct of each sample was
155   calculated based on it. Then, *ΔΔ*Ct for each condition was calculated on the basis of the RT-

156 qPCR result for 0 h inoculation under each condition. Moreover, the results of RT-qPCR analysis
157 were clustered using the unweighted pair group method with arithmetic mean (UPGMA) and
158 output to heat maps using *R* (ver. 3.5.1) software with two packages (*Heatplus*, and *viridis*).
159
160 **PCR primers**
161 Primers were designed and tested for the following: coronatine synthesis-related genes (*cfl*, *cfa1*,
162 *cfa5*, *cfa9*, *cmaD*, *cmaU*, *corR*, *corS,* and *corP*); phaseolotoxin synthesis-related genes (*argK* and
163 *argD*); and type III effector and related genes (*avrD1*, *avrE1*, *avrpto5*, *avrRpm1*, *avrRps4*,
164 *hopAA1-1*, *hopAE1*, *hopAH1*, *hopAI1*, *hopAS1*, *hopAU1*, *hopAZ1*, *hopD1*, *hopJ*, *hopM1*, *hopN*,
165 *hopQ1*, *hopR1*, *hopS2*, *hopY1*, *hopZ3*, *hrpK,* and *hrpL*) (Table S1). The primers for the five
166 selected reference genes (details above) were also designed. These were "50S ribosomal protein
167 L13", "bifunctional glutamine synthetase adenylyltransferase/deadenyltransferase", "tRNA
168 dihydrouridine (20/20a) synthase *dusA*", "transcriptional regulator *ftrA*", and "microcin ABC
169 transporter ATP-binding protein". It was confirmed that these primer sets can be applied not only
170 to Psa6 but also to Psa1 (excluding the coronatine gene) and Psa3 (excluding the two phytotoxin
171 genes) equally (Fig S1). The similar primer sets were also designed for the Psg homolog genes
172 (Table S1).
173   To confirm the validity of the designed primers, the extracted DNA was subjected to PCR
174 using Takara Ex Taq Hot Start Version (Takara Bio) with the following conditions: denaturation
175 at 95 ºC for 10 min; a cycle of 95 ºC for 30 s, 65 ºC for 30 s, and 72 ºC for 1 m, repeated 40
176 times; and extension at 72 ºC for 7 m. It was confirmed that respective amplicons were single
177 bands by electrophoresis (Fig S1), and each melt peak of single band using melt curve analysis
178 was also measured (Table S1). In addition, each amplification efficiency of primer sets was
179 calculated (Table S1). These appropriate primer sets were used for RT-qPCR analysis.
180
181
182 **Results and Discussion**
183 **Gene expression of Psa6 under different culture conditions using RNA-Seq analysis**
184 RNA-Seq data of Psa6 was archived in NCBI GEO (accession no. GSE149743). RNA-Seq of
185 Psa6 revealed the behavior of 5,796 genes (Table S2). The expression values from the HS
186 medium at 27 ºC were used to calculate the expression values from the HS medium at 18 °C and
187 the HSC medium at 27 and 18 ºC. Seventy-eight genes had an FDR value lower than 0.1 (Table
188 1). In contrast to the HS medium at 27 ºC, 26 genes were induced in the HSC medium at 18 ºC,
189 including the type III chaperone ShcN protein gene and the type III effector (translocator) *hrpK*.
190 In particular, *hrpK* had a large effect size (0.96). However, there was no significant induction of
191 other type III effector genes. In addition, neither the induction nor the suppression of genes
192 involved in toxin production was observed. In contrast to HS medium at 27 ºC, 17 genes were
193 suppressed in both the HSC medium and the 18 ºC condition; however, no genes thought to be
194 involved in virulence were significant (Table 2). Similarly, no virulence genes were significantly
195 induced or suppressed under the different culture conditions. The HSC medium is an

196   improvement of the HS medium and induces coronatine production (*Palmer & Bender, 1993*).
197   However, we found no significant change in the expression of phytotoxin genes. This suggests
198   limited coronatine synthesis in the 3 h incubation of Psa6. Incubation of *P. syringae* at 18 °C can
199   induce phytotoxin production compared with incubation at 27 °C (*Nüske & Fritsche, 1989*;
200   *Palmer & Bender, 1993*), but we did not observe significant induction of phytotoxins under those
201   conditions (Table 2). This suggests that temperature in a 3 h incubation may have little effect on
202   phytotoxin production for Psa6. To clarify the role of temporal changes in the behavior of
203   virulence genes, such as type III effector genes and phytotoxin synthesis genes, we examined the
204   behavior of gene expression over time using RT-qPCR analysis. Because the relative expression
205   values in RT-qPCR analyses are calculated using constantly expressed reference genes, the
206   selection of appropriate reference genes is important. Based on the conditions described in the
207   Materials and Methods section, we selected five candidates among a total of 5,796 genes (Table
208   S3). From these five genes (50S ribosomal protein L13, bifunctional glutamine synthetase
209   adenylyltransferase/deadenyltransferase, tRNA dihydrouridine (20/20a) synthase *dusA*,
210   transcriptional regulator *ftrA*, and microcin ABC transporter ATP-binding protein), three genes
211   for each bacterium were used as reference genes. For these genes, expression level was high and
212   stable (expression change was low), regardless of the media used. Thus, we designed primers for
213   the RT-qPCR analysis based on the sequences of the following reference genes: 50S ribosomal
214   protein L13, bifunctional glutamine synthetase adenylyltransferase/deadenyltransferase, and
215   tRNA dihydrouridine (20/20a) synthase *dusA* for Psa6; 50S ribosomal protein L13, bifunctional
216   glutamine synthetase adenylyltransferase/deadenyltransferase, and transcriptional regulator *ftrA*
217   for Psa1 and Psa3; and 50S ribosomal protein L13, bifunctional glutamine synthetase
218   adenylyltransferase/deadenyltransferase, and microcin ABC transporter ATP-binding protein for
219   Psg (Table S1). Since these reference genes are a group of genes with stable expression and
220   housekeeping, it is expected that strains other than Psa6 have similar expression tendencies. In
221   addition, it is expected that internal control can be guaranteed by the geometric mean of 3 kinds
222   of genes in order to avoid the bias of expression of each gene.
223
224   **Temporal changes in the expression of virulence genes of Psa6 in RT-qPCR analysis**
225   The expression of phytotoxin synthesis genes and type III effector genes of Psa6 was
226   investigated with RT-qPCR. Normalization was performed using the reference genes selected
227   above.
228   For the reference genes, the amplification efficiency (*E*) of target genes in qPCR was
229   approximately 2.0 (Table S1) and three genes were used as the reference (*n* = 3). Thus, using
230   equation (1) in the Materials and Methods section with each *E* of primer sets, the relative
231   expression levels were calculated from the Ct value at a certain time and the Ct value at 0h. Here,
232   in order to indicate that the results of RNA-Seq and the results of RT-qPCR did not diverge
233   significantly, the expression levels of the virulence genes in the RT-qPCR analysis for Psa6 and
234   the expression levels of the same genes in the RNA-Seq analysis were compared (Table S4).
235   When the expression levels of the virulence genes from RNA-Seq were plotted on the vertical

236   axis and those from RT-qPCR were plotted on the horizontal axis, both RNA-Seq and RT-qPCR
237   results were correlated (Fig. 1). The plots were concentrated in the first and third quadrants (R^2
238   (coefficient of determination) value is about 0.6), although some genes were expressed
239   differently. We concluded that the results from RT-qPCR can almost function as a representation
240   of transcriptome analysis.
241   The expression of phytotoxin synthesis genes and effector-related genes of Psa6 under
242   various culture conditions with different incubation times was investigated using RT-qPCR
243   (Table S5 & Data S1). From the results of RT-qPCR analysis, heat maps were output (Fig. 2).
244   We found that phytotoxin synthesis genes and effector genes of Psa6 were induced in HS and
245   HSC media (Fig. 2A), which are known to induce coronatine production. In contrast, these genes
246   were not induced in the *hrp*-inducing medium, which is known to induce *hrp*-dependent effector
247   genes. The LB and *hrp*-inducing media were clustered together and were separate from the HS
248   and HSC media. In the LB and *hrp*-inducing media cluster, most genes were suppressed.
249   Moreover, in the *hrp*-inducing medium, no clear induction of the *hrp*L-dependent effector genes
250   of Psa6 was observed. On the other hand, phytotoxin synthesis genes and effector genes were
251   significantly induced in HS and HSC media. Additionally, incubation at 18 ºC and 27 ºC did not
252   result in significant changes to gene expression; samples from each temperature were clustered
253   separately. This suggests that culture medium composition and incubation time have a stronger
254   effect on Psa6 cultivation than incubation temperature.
255   Intriguingly, the expression of virulence genes was generally separated into the following two
256   groups: early-inducible genes and late-inducible genes (Fig. 2). This was more clearly observed
257   in the heat map, excluding the conditions of *hrp*-inducing medium (Fig. 2B). The early-inducible
258   genes (*hrpL*, *avrD1*, *avrpto5*, *avrRpm1*, *hopAA1-1*, *hopAE1*, *hopAU1*, *hopAZ1*, *hopM1*, *hopN1*,
259   *hopQ1*, *hopR1*, *hopS2*, *hopY1*, *hrpK,* and *argK*) were induced after 3 h of incubation but
260   suppressed at 6 and 12 h. Most of them were thought to be *hrpL*-dependent effector genes (*Lam
261   et al., 2014*; *Vencato et al., 2006*). The early-inducible genes of Psa6 contains *hrpL* and several
262   effector genes. It is known that the HrpL protein binds to the Hrp box promoter sequence on the
263   upper region of protein-encoding sequences in several effector genes (Greenberg & Yao, 2004;
264   Lam et al., 2014), then, we could also find the Hrp box promoter sequences in some early-
265   inducible genes of Psa6 (Fig S2). Therefore, HrpL regulation may be involved in the early-
266   inducible genes of Psa6 in response to HS and HSC media. However, further research is needed
267   on this detail. In contrast, the late-inducible genes (*avrE1*, *avrRps4*, *hopAH1*, *hopAI1*, *hopAS1*,
268   *hopD1*, *hopJ*, *hopZ3*, *cfl*, *cfa1*, *cfa5*, *cfa9*, *cmaD*, *cmaU*, *corP*, *corR*, *corS,* and *argD*) were
269   induced after 6 h of incubation. These genes included phytotoxin synthesis genes, except *argK*
270   and some effector genes. Thus, when Psa6 is incubated under phytotoxin-induced conditions (HS
271   and HSC media), induction of phytotoxin synthesis genes followed that of effector genes. In
272   addition, phytotoxin synthesis genes were expressed for longer than the *hrpL*-regulated effectors.
273
274   **Comparison of gene expression behavior between Psa6 and other bacteria**

275 As described above, in Psa6, the effector genes are not induced in the *hrp*-inducing medium, and
276 the induction of virulence genes in phytotoxin-inducing media is strictly controlled over time. It
277 was unclear whether these behaviors of gene expression were Psa6 specific; thus, similar tests
278 were performed on the other bacteria. The expressions of virulence genes in two biovars (Psa1
279 and Psa3) and one other species (Psg) were investigated using RT-qPCR (Table S6 & Data S2).
280 Normalization was performed using the reference genes selected above. The expression level of
281 virulence genes was compared across all bacteria. Heat maps were generated from the expression
282 patterns of the effector genes shared by each bacterium (Fig. 3). Psa1 and Psa3 were nested in
283 the heat map (Fig. 3A). In Psa6, the expression of effector genes was significantly induced
284 compared with other bacteria. Furthermore, Psg was scattered in clusters of Psa1 and Psa3, and
285 the clusters did not split due to differences in pathovars. When clustering was performed with
286 only common effectors of both Psa1 and Psa3 (omitting Psa6 and Psg), medium composition had
287 no effect on gene expression (Fig. 3B). Our results show that medium composition and
288 incubation time did not significantly affect the expression of effector genes in Psa1, Psa3, or Psg.
289   We also compared phytotoxin synthesis genes of Psa6 with other bacteria. To compare
290 the expression of coronatine synthesis genes in Psa6 and Psg, the expression levels of *cfa1* and
291 *cmaD* at 0, 3, and 6 h incubation were examined (Table 3). The results showed that incubation of
292 Psa6 in HS and HSC media induced the expression of *cfa1* and *cmaD* over time, but incubation
293 in LB and *hrp*-inducing media significantly suppressed *cfa1* and slowly induced the expression
294 of *cmaD*. Therefore, the behavior of the coronatine synthesis genes of Psa6 was affected by the
295 medium composition. On the other hand, Psg was not affected by medium composition. In Psg,
296 there were no differences in gene expression across the different media, and only *cfa1* was
297 induced over time regardless of medium composition. There was no induction or suppression of
298 *cmaD*. Additionally, we compared the expression of phaseolotoxin synthesis-related genes in
299 Psa6 and Psa1 (Table 4). In Psa6, the expression of *argK* and *argD* was slowly induced by
300 culture in HS and HSC media. In addition, the expression of *argK* was notably induced and *argD*
301 was greatly suppressed in LB and *hrp*-inducing media. However, in Psa1, *argK* was induced in
302 LB and *hrp*-inducing media, and *argD* remained expressed at a constant level without induction
303 or suppression. In summary, the coronatine and phaseolotoxin synthesis genes in Psa6 differed in
304 their response to medium composition and incubation time compared to other bacteria, with a
305 particularly strong effect of medium composition.
306
307 **Expression patterns and conditions of the virulence genes in Psa6**
308 Coronatine production in Psg PG 4180 is more active in cultures at 18 ºC than 24 ºC or higher
309 (*Palmer & Bender, 1993*). In contrast, coronatine production in *P. syringae* pv. *tomato* (Pto) DC
310 3000 is temperature independent and constant (*Braun et al., 2008*). Additionally, seven strains of
311 *P. syringae*, including Psg PG 4180, Psg 7a, and Pto PT 23.2, produce more coronatine in HSC
312 medium than in HS medium (*Palmer & Bender, 1993*). However, two strains of *P. syringae* pv.
313 *maculicola* 438 and Pst DC 3000 do not produce coronatine in HSC medium (*Palmer & Bender,*
314 *1993*). Moreover, in Psg PG 4180, the CmaB protein, a synthesis protein of coronatine, decreases

315 4 h after the temperature is changed from 18 ºC to 28 ºC, suggesting that changes in gene
316 expression can be observed at an earlier stage (*Budde et al., 1998*). However, the coronatine
317 synthesis genes of Psa6 were induced later, regardless of temperature (Fig. 2). Thus, the
318 coronatine synthesis genes of Psa6 may be regulated by different environmental conditions than
319 Psg PG 4180. In addition, in the strain of Psg used in this study (MAFF 301683), which was
320 isolated in Japan, the expression of the coronatine synthesis genes was induced regardless of
321 medium composition (Table 3). Conversely, in a different Psg strain, PG 4180, coronatine
322 synthesis was induced in HS and HSC media (*Palmer & Bender, 1993*). Thus, depending on the
323 type of *Pseudomonas* (pathovars and strains), coronatine production is thought to be affected by
324 various environmental conditions.
325 Moreover, despite Psa6 and Psa1 possessing a similar phaseolotoxin synthesis gene cluster
326 (*Fujikawa & Sawada, 2019*), both expressed them differently (Table 4). This gene cluster is
327 contained in the *tox* island in the genomes of Psa6 and Psa1. However, in Psa6, the
328 *ginABCD* operon, which is located at the left end of the *tox* island and is involved in the
329 excision/insertion of the *tox* island, was truncated, suggesting that the island of biovar 6 might
330 have lost its mobility (*Fujikawa & Sawada, 2019*). At present, it is unknown whether the
331 expression pattern of the phaseolotoxin synthesis genes is different due to differences in the
332 *ginABCD* operon or whether it depends on another regulation system. This requires further
333 investigation.
334 We found that Psa6 did not induce *hrpL*-dependent effectors in the *hrp*-inducing medium but
335 was induced in HS and HSC media (Fig. 2). The *hrp*-inducing medium is an oligotrophic
336 medium (*Huynh et al., 1989*), but HS and HSC media contain rich sugars and minerals (*Palmer
337 & Bender, 1993*). In Psa6, phytotoxin production and effector-related genes were induced
338 similarly even with different concentrations of glucose (10 g/L in HS medium and 20 g/L in HSC
339 medium) (Table 1). However, fructose (1.8 g/L) in the *hrp*-inducing medium was not
340 significantly induced. This may be due to qualitative or quantitative differences in carbon
341 sources. Additionally, it seemed that the FeCl$_3$ concentration (HS, HSC, and the *hrp*-inducing
342 media) (Table 1) may not be involved in the expression behavior of phytotoxin synthesis genes
343 and effector genes. In this study, HS and HSC media were found to induce the virulence genes of
344 Psa6. These genes were divided into two groups according to how long it took for them to be
345 induced.
346 The early-inducible genes included many effectors and *hrpL*. The effectors in this group are
347 involved in plant immunity such as pathogen-associated molecular patterns (PAMPs) triggered
348 immunity (PTI) and effector-triggered immunity (ETI) (*Jones & Dangl, 2006*). PTI is a plant
349 immune system that responds to the cellular components carried by pathogens such as flagella
350 protein and elongation factor. A plant immune system activated by PTI induces various
351 antimicrobial molecules such as salicylic acid and PR (pathogenesis-related) proteins, resulting
352 in the inhibition of pathogen spread. In response, pathogens can interfere with PTI by injecting
353 the effectors to avoid the plant defense. However, plants have ETI, another plant immune
354 system, which responds to the effectors. Thus, the plant immune system is expressed as a zigzag

355 model (*Jones & Dangl, 2006*). Although the function of these effectors in Psa6 to the host plants
356 is unknown, these homologous proteins have been reported as follows; AvrPto5, contained in the
357 early-inducible gene of Psa6, induces PTI, whereas HopS, HopAA1, and HopM1 inhibit PTI. In
358 addition, AvrPto5, AvrRpm1, and HopD induce ETI, whereas HopD, HopN, and HopS suppress
359 ETI (*Lindeberg et al., 2012*).
360   In contrast to the early-inducible genes, the late-inducible genes included phytotoxin
361 synthesis-related genes other than *argK*. Because it has been reported that coronatine and other
362 phytotoxins accumulate over time (*Palmer & Bender, 1993*), phytotoxin synthesis-related genes
363 may require longer periods than most effector genes for expression. On the other hand, the *argK*
364 gene of Psa6 was an early inducible gene. Because *argK* is also known to be involved in the
365 production of phaseolotoxin-insensitive ornithine carbamoyltransferase (*Sawada et al., 1997*),
366 this early expression may lead to resistance to phaseolotoxin and subsequent phaseolotoxin
367 production. Additionally, some effector genes were listed in the late-inducible genes. It is known
368 that AvrE and AvrRps4 in Pto suppress PTI (*Lindeberg et al., 2012*), while HopZ3, AvrRps4,
369 HopAS1, and HopAI1 induce ETI (*Lewis et al., 2014*; *Lindeberg et al., 2012*; *Sohn, Zhang &
370 Jones, 2009*; *Sohn et al., 2012*), and HopZ suppresses ETI in *Arabidopsis* (*Lindeberg et al.,
371 2012*). These late-inducible genes in Psa6 may also trigger ETI, such as the Pto-*Arabidopsis*
372 interaction. However, it is thought to function under conditions different from those of the early-
373 inducible genes, for example, at different times, in different environment, and with different
374 hosts.
375   The coronatine synthesis genes of Psa6 are located on plasmids whose sequences are
376 highly homologous to those of Psa2 and Psg (*Fujikawa & Sawada, 2019*). The phaseolotoxin
377 production genes of Psa6 are located on the *tox* island in the genome, and it is thought that the
378 region was originally in another bacterium and transferred to Psa1 and Psa6 through horizontal
379 gene transfer (*Sawada, 2016*; *Fujikawa & Sawada, 2019*). In addition, along with the presence or
380 absence of virulence factors, it was suggested that the regulation of gene expression varied also
381 among pathovars and biovars. Phytopathogenic bacteria are then thought to have coevolved with
382 plants, with their behavior and infection method related to the life cycle of the host plant. Psa6,
383 with two phytotoxins, was unique not only in its physiological characteristics but also in the
384 expression behavior of phytotoxin synthesis genes and effector genes. Although the relationship
385 between the number of virulence factors and their expression behavior is still not clear, we
386 propose that strict gene expression is thought to have been acquired with the acquisition of many
387 virulence factors. The behavior of virulence genes in Psa clarified in this study will help
388 elucidate the differences in virulence and survival strategies among biovars.
389

390 **Conclusions**
391 *Pseudomonas syringae* pv. *actinidiae* biovar 6 (Psa6) is a causal agent of kiwifruit bacterial
392 canker and is a unique plant pathogenic bacterium, producing two types of phytotoxins,
393 coronatine and phaseolotoxin. We investigated the expression behavior of virulent genes of Psa6
394 under various culture conditions. The expression pattern of phytotoxin synthesis genes in Psa6

395 was different from that of another pathovar, *P. s.* pv. *glycinea*, which produces coronatine, and
396 another biovar, Psa1, which produces phaseolotoxin. In addition, the expression of virulence
397 genes (phytotoxin synthesis genes and type III effector genes) was induced in the coronatine-
398 producing media regardless of temperature, and confirmed that the expression behavior had two
399 patterns: early-inducible and late-inducible (Fig. 4). The expression behavior of virulence genes
400 of Psa6 clarified in this study will contribute to elucidating the virulence of Psa to kiwifruits and
401 survival strategies among Psa biovars and plant pathogenic *Pseudomonas*.

402
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References

414 Bender CL 1999. Chlorosis-inducing phytotoxins produced by *Pseudomonas syringae. Eur J*
415   *Plant Pathol* 105(1): 1–12 DOI 10.1023/A:1008692227307.
416 Bertani G 1951. Studies on lysogenesis. I. the mode of phage liberation by lysogenic
417   *Escherichia coli. J Bacteriol.* 62(3): 293–3000.
418 Braun Y, Smirnova AV, Schenk A, Weingart H, Burau C, Muskhelishvili G, Ullrich MS. 2008.
419   Component and protein domain exchange analysis of a thermoresponsive, two-
420   component regulatory system of *Pseudomonas syringae. Microbiology* 154(9): 2700–
421   2708 DOI 10.1099/mic.0.2008/018820-0.
422 Budde IP, Rohde BH, Bender CL, Ullrich MS. 1998. Growth phase and temperature influence
423   promoter activity, transcript abundance, and protein stability during biosynthesis of the
424   *Pseudomonas syringae* phytotoxin coronatine. *Journal of bacteriology* 180(6): 1360–
425   1367.
426 Chapman JR, Taylor RK, Weir BS, Romberg MK, Vanneste JL, Luck J, Alexander BJ. 2012.
427   Phylogenetic relationships among global populations of *Pseudomonas syringae* pv.
428   *actinidiae. Phytopathology* 102(11): 1034-1044 DOI 10.1094/PHYTO-03-12-0064-R.
429 Coombes BK. 2009. Type III secretion systems in symbiotic adaptation of pathogenic and non-
430   pathogenic bacteria. *Trends Microbiol* 17(3): 89–94 DOI 10.1016/j.tim.2008.11.006.
431 Fujikawa T, Sawada H. 2016. Genome analysis of the kiwifruit canker pathogen *Pseudomonas*
432   *syringae* pv. *actinidiae* biovar 5. *Sci Rep* 6: 21399 DOI 10.1038/srep21399.
433 Fujikawa T, Sawada H. 2019. Genome analysis of *Pseudomonas syringae* pv. *actinidiae* biovar
434   6, which produces the phytotoxins, phaseolotoxin and coronatine. *Sci Rep* 9: 3836 DOI
435   10.1038/s41598-019-40754-9.
436 Geng X, Jin L, Shimada M, Kim MG, Mackey D. 2014. The phytotoxin coronatine is a

437   multifunctional component of the virulence armament of *Pseudomonas syringae*. *Planta*
438   240(6): 1149–1165 DOI 10.1007/s00425-014-2151-x.
439   Greenberg JT, Yao N. 2004. The role and regulation of programmed cell death in plant-pathogen
440   interactions. *Cell Microbiol* 6(3): 201-211 DOI 10.1111/j.1462-5822.2004.00361.x.
441   Hoitink HA, Sinden SL. 1970. Partial purification and properties of chlorosis inducing toxins of
442   *Pseudomonas phaseolicola* and *Pseudomonas glycinea*. *Plant Pathology* 60(8): 1236-
443   1237 DOI 10.1094/Phyto-60-1236.
444   Huynh TV, Dahlbeck D, Staskawicz BJ. 1989. Bacterial blight of soybean: regulation of a
445   pathogen gene determining host cultivar specificity. *Science* 245(4924): 1374–1377 DOI
446   10.1126/science.2781284.
447   Jones JD, Dangl JL. 2006. The plant immune system. *Nature* 444: 323–329 DOI
448   10.1038/nature05286.
449   Lam HN, Chakravarthy S, Wei HL, BuiNguyen H, Stodghill PV, Collmer A, Swingle BM,
450   Cartinhour SW. 2014. Global analysis of the HrpL regulon in the plant pathogen
451   *Pseudomonas syringae* pv. *tomato* DC3000 reveals new regulon members with diverse
452   functions. *PLoS One* 9(8): e106115 DOI 10.1371/journal.pone.0106115.
453   Lewis JD, Wilton M, Mott GA, Lu W, Hassan JA, Guttman DS, Desveaux D. 2014.
454   Immunomodulation by the *Pseudomonas syringae* HopZ type III effector family in
455   *Arabidopsis*. *PLoS One*. 2014 Dec 9(12): e116152 DOI 10.1371/journal.pone.0116152.
456   Lindeberg M, Cunnac S, Collmer A. 2012. *Pseudomonas syringae* type III effector repertoires:
457   last words in endless arguments. *Trends Microbiol* 20(4): 199-208 DOI
458   10.1016/j.tim.2012.01.003.
459   Mitchell RE. 1976. Isolation and structure of a chlorosis inducing toxin of *Pseudomonas
460   phaseolicola*. *Phytochemistry* 15(12): 1941–1947 DOI 10.1016/S0031-9422(00)88851-2.
461   Nüske J, Fritsche W. 1989. Phaseolotoxin production by *Pseudomonas syringae* pv.
462   *phaseolicola:* the influence of temperature. *J Basic Microbiol* 29(7): 441-447 DOI
463   10.1002/jobm.3620290713.
464   Palmer DA, Bender CL. 1993. Effects of environmental and nutritional factors on production of
465   the polyketide phytotoxin coronatine by *Pseudomonas synrngae* pv. *glycinea*. *Appl
466   Environ Microbiol.* 59(5): 1619-1626.
467   Patil SS, Youngblood P, Christiansen P, Moore RE. 1976. Phaseotoxin A. an antimetabolite from
468   *Pseudomonas phaseolicola*. *Biochem Biophys Res Commun* 69(4): 1019–1027 DOI
469   10.1016/0006-291X(76)90474-5.
470   Sawada H, Takeuchi T, Matsuda I. 1997. Comparative analysis of *Pseudomonas syringae* pv.
471   *actinidiae* and pv. *phaseolicola* based on phaseolotoxin-resistant ornithine
472   carbamoyltransferase gene (*argK*) and 16S-23S rRNA intergenic spacer sequences. *Appl
473   Environ Microbiol* 63(1): 282–288.
474   Sawada H. 2014. Studies on the classification and evolution of phytopathogenic bacteria of the
475   genera *Agrobacterium* and *Pseudomonas*. [in Japanese] *Jpn. J. Phytopathol.* 80: 139–
476   142.
477   Sawada H. 2016. Characteristics of pathogens causing bacterial canker of kiwifruit in Japan. [in
478   Japanese] *Annual Report on Exploration and Introduction of Microbial Genetic
479   Resources* 25: 103–110.
480   Sawada H, Fujikawa T. 2019a. Genetic diversity of *Pseudomonas syringae* pv. *actinidiae*,
481   pathogen of kiwifruit bacterial canker. *Plant Pathol.* 68: 1235-1248 DOI
482   10.1111/ppa.13040.

483   Sawada H, Fujikawa T. 2019b. Genetic diversity among biovars of kiwifruit canker pathogen
484   *Pseudomonas syringae* pv. *actinidiae*. [in Japanese, English summary] *Jpn. J.*
485   *Phytopath.* 85(1): 3-17 DOI 10.3186/jjphytopath.85.3.
486   Smith A, Lovelace AH, Kvitko BH. 2018. Validation of RT-qPCR approaches to monitor
487   *Pseudomonas syringae* gene expression during infection and exposure to pattern-
488   triggered immunity. *Mol Plant Microbe Interac*t 31(4): 410-419 DOI 10.1094/MPMI-11-
489   17-0270-TA.
490   Sohn KH, Saucet SB, Clarke CR, Vinatzer BA, O'Brien HE, Guttman DS, Jones JD. 2012.
491   HopAS1 recognition significantly contributes to *Arabidopsis* nonhost resistance to
492   *Pseudomonas syringae* pathogens. *New Phytol* 193(1): 58-66 DOI 10.1111/j.1469-
493   8137.2011.03950.x.
494   Sohn KH, Zhang Y, Jones JD. 2009. The *Pseudomonas syringae* effector protein, AvrRPS4,
495   requires in planta processing and the KRVY domain to function. Plant J 57(6): 1079–
496   1091 DOI 10.1111/j.1365-313X.2008.03751.x.
497   Strange RN. 2007. Phytotoxins produced by microbial plant pathogens. *Nat Prod Rep* 24: 127–
498   144 DOI 10.1039/B513232K.
499   Takikawa Y, Serizawa S, Ichikawa T. 1989. *Pseudomonas syringae* pv. *actinidiae* pv. *nov.:* the
500   causal bacterium of canker of kiwifruit in Japan. *Ann Phytopath Soc Japan* 55: 437-444.
501   Tamura A, Imamura M, Yoneyama K, Kohno Y, Takikawa Y, Yamaguchi I. 2002. Role of
502   phaseolotoxin production by *Pseudomonas syringae* pv. *actinidiae* in the formation of
503   halo lesions of kiwifruit canker disease. *Physiol Mol Plant Pathol* 60(4): 207–214 DOI
504   10.1006/pmpp.2002.0405.
505   Ushiyama K. 1993. Studies on the epidemics and control of bacterial canker of kiwifruitcaused
506   by *Pseudomonas syringae* pv. *actinidiae*. [in Japanese, English summary] *Bulletin of the*
507   *Kanagawa Horticultural Experiment Station* 43: 1-76 DOI 10.11501/3095787.
508   Vencato M, Tian F, Alfano JR, Buell CR, Cartinhour S, DeClerck GA, Guttman DS, Stavrinides
509   J, Joardar V, Lindeberg M, Bronstein PA, Mansfield JW, Myers CR, Collmer A, Schnider DJ.
510   2006. Bioinformatics-enabled identification of the HrpL regulon and type III secretion system
511   effector proteins of *Pseudomonas syringae* pv. *phaseolicola* 1448A. *Mol Plant Microbe*
512   *Interact* 19(11): 1193-1206 DOI: 10.1094/MPMI-19-1193

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515   **Figure legends**
516   **Figure 1. Comparison of expression trends in the RNA-seq and RT-qPCR analyses.** The
517   expression levels of the virulence genes in RNA-Seq were plotted on the vertical axis and that in
518   RT-qPCR were plotted on the horizontal axis. Most of the plots were concentrated in the first
519   and third quadrants, while some were concentrated in the second and fourth quadrants . The
520   yellow wavy line indicates a linear approximation, and R^2 value (about 0.6) was obtained.
521
522   **Figure 2. Heat map constructed from the expression level of virulence genes of Psa6 under**
523   **each culture condition.** The results of RT-qPCR analysis of Psa6 were clustered using the
524   unweighted pair group method with arithmetic mean (UPGMA). **A**, Heat map of expression
525   levels of virulence genes under each culture condition. **B**, Reconstructed heat map excluding the
526   conditions of the *hrp*-inducing medium. Gene names highlighted in blue are phaseolotoxin

527 synthesis genes, and gene names highlighted in yellow are coronatine synthesis genes.
528 Remaining genes are type III effectors. In heat maps, induced genes are indicated as yellow
529 boxes and suppressed genes are indicated as blue boxes.
530
531 **Figure 3. Heat map constructed from the expression levels of virulence genes of Psa6, Psa1,**
532 **Psa3, and Psg under each culture condition.** The results of RT-qPCR analysis of Psa6, Psa1,
533 Psa3, and Psg were clustered using the unweighted pair group method with arithmetic mean
534 (UPGMA). **A**, Heat map of expression levels of virulence genes under each culture condition. **B**,
535 Reconstructed heat map excluding the results of both Psa6 and Psg. In heat maps, induced genes
536 are indicated as yellow boxes and suppressed genes are indicated as blue boxes.
537
538 **Figure 4. Schematic diagram of the expression pattern of Psa6 virulence genes in different**
539 **media.** The expression of virulence genes (type III effector genes and phytotoxin synthesis
540 genes) of Psa6 was induced in the coronatine-producing media, and the expression behavior had
541 two patterns: early-inducible and late-inducible. These genes were suppressed or constant in LB
542 medium and the *hrp*-inducing medium.

Each composition of media used in this study

1 **Table 1. Each composition of media used in this study**

Per liter of medium	HS	HSC	*hrp*-inducing	LB
NH_4Cl	1.0g	1.0g		
$(NH_4)_2SO_4$			1.0g	
$MgCl_2 \cdot 6H_2O$			0.34g	
$MgSO_4 \cdot 7H_2O$	0.3g	0.2g		
KH_2PO_4	4.1g	4.1g	6.8g	
K_2HPO_4	3.6g	3.6g		
KNO_3		0.3g		
NaCl			0.1g	10g
D-glucose	10g	20g		
D-fructose			1.8g	
$FeCl_3 \cdot 6H2O$	2μM	20μM	20μM	
Yeast extract				5.0g
Bactopeptone				10g

List of gene expression of Psa6 in difference medium conditions by RNA-Seq analysis (FDR <0.1 with effect size)

1   **Table 2. List of gene expression of Psa6 in difference medium conditions by RNA-Seq analysis (FDR <0.1**
2   **with effect size)**

ID	product	27HSC[a]	18HS[b]	18HSC[c]	logCPM	PValue	FDR	Effect size
BUE60_04820	heat-shock protein	1.355338	-2.43875	-1.31508	11.73123	2.29E-11	1.33E-07	0.825882
BUE60_03120	transposase	1.020947	-9.70426	0.793676	11.37198	5.78E-11	1.68E-07	0.641125
BUE60_14130	transposase	0	0	9.353037	8.873037	8.51E-10	1.64E-06	0.59108
BUE60_20545	peptidase M4	2.489317	4.443465	4.633089	9.090263	4.33E-08	6.26E-05	0.843105
BUE60_04770	hypothetical protein	-0.34373	-7.61338	-7.61338	8.059493	5.40E-08	6.26E-05	0.670986
BUE60_01870	phage tail protein	7.758212	6.587112	7.860501	8.547592	3.98E-07	0.000384	0.541496
BUE60_06970	hypothetical protein	-2.58953	-0.8029	-8.1928	8.386577	1.29E-06	0.001067	0.864256
BUE60_14400	4a-hydroxytetrahydrobiopterin dehydratase	7.494481	7.602294	8.036372	8.691885	2.15E-06	0.001554	0.56063
BUE60_02835	chemotaxis protein	2.250013	1.336789	2.235526	11.70462	2.82E-06	0.001818	0.626422
BUE60_17905	DNA-directed DNA polymerase	0	9.167101	6.180072	8.399849	3.17E-06	0.001838	0.721365
BUE60_02480	phospholipid-binding protein	2.75255	2.175321	2.667653	11.94782	4.11E-06	0.001985	0.799186
BUE60_04310	hypothetical protein	0.224221	-7.50516	-7.50516	8.207357	3.77E-06	0.001985	0.637833
BUE60_16610	type III effector (translocator) HrpK	0.606558	2.038242	1.196631	12.13895	4.71E-06	0.002099	0.957299
BUE60_04320	ribosomal subunit interface protein	1.726174	0.111266	1.619895	11.57393	8.20E-06	0.003394	0.768705
BUE60_28960	transposase	0	9.007053	7.799665	8.680074	1.35E-05	0.004903	0.403141
BUE60_03115	transposase	-0.59594	0.589465	-9.83131	10.6735	1.30E-05	0.004903	0.476177
BUE60_23710	ester cyclase	0.775814	-6.16163	-6.16163	7.36076	2.09E-05	0.006918	0.599137
BUE60_21600	transporter	0	6.717301	7.851922	7.885333	2.15E-05	0.006918	0.367771
BUE60_05090	ATP-dependent chaperone ClpB	1.973675	-0.24943	0.635359	9.808781	2.36E-05	0.007214	0.855973
BUE60_16865	hypothetical protein	0	7.452753	0	6.847248	2.67E-05	0.00775	0.834247
BUE60_14380	acetate--CoA ligase	-0.39884	-3.76361	-3.20795	8.443014	3.68E-05	0.010001	0.773293
BUE60_12810	Fe/S-dependent 2-methylisocitrate dehydratase AcnD	-2.44697	-3.73661	-4.31049	7.889943	3.80E-05	0.010001	0.840004
BUE60_09615	50S ribosomal protein L11	0.386928	0.149426	-6.90695	8.113202	5.79E-05	0.014595	0.523026
BUE60_02595	molecular chaperone DnaK	1.635999	-0.18151	0.473407	11.0735	6.45E-05	0.015578	0.932595

BUE60_02495	30S ribosomal protein S15	-0.67634	-3.71221	-3.52621	12.0987	7.41E-05	0.017175	0.614689
BUE60_21040	ribosomal-protein-alanine N-acetyltransferase	2.254871	1.463555	-5.13538	7.73098	7.73E-05	0.01723	0.854178
BUE60_24615	threonine ammonia-lyase, biosynthetic	-2.31336	-7.75066	-2.51696	7.85215	8.26E-05	0.017737	0.580708
BUE60_14740	hypothetical protein	-6.33963	-6.33963	0.83639	7.541523	8.97E-05	0.018564	0.670119
BUE60_19565	hypothetical protein	1.419291	2.063655	2.243516	10.73711	0.000108	0.021543	0.754757
BUE60_17855	hypothetical protein	1.644416	0.041077	0.333665	10.61235	0.000112	0.02158	0.934538
BUE60_15390	gamma carbonic anhydrase family protein	0.672182	-0.73084	-6.19487	7.496001	0.000115	0.02158	0.722079
BUE60_02340	transposase	-5.51749	3.725977	1.970766	8.817886	0.000128	0.023196	0.714283
BUE60_12815	2-methylcitrate synthase	-3.05674	-7.53602	-3.3801	7.531794	0.000132	0.023209	0.872486
BUE60_18535	ribosomal subunit interface protein	1.422023	-0.98943	0.057456	10.76842	0.000151	0.025747	0.757798
BUE60_19430	hypothetical protein	1.211633	-5.7711	-5.7711	7.283092	0.000158	0.0261	0.73941
BUE60_13595	hypothetical protein	1.636492	-5.54256	-5.54256	7.34782	0.000186	0.027557	0.412538
BUE60_21160	hypothetical protein	0.130148	-6.04703	-6.04703	6.997812	0.00019	0.027557	0.777151
BUE60_19345	glutathione S-transferase	0	6.553643	7.982156	7.93946	0.000185	0.027557	0.497624
BUE60_15180	hypothetical protein	-0.64369	-6.95686	-6.95686	7.415176	0.000178	0.027557	0.528899
BUE60_07695	Fis family transcriptional regulator	-6.07158	0.083934	-6.07158	6.817511	0.000184	0.027557	0.867404
BUE60_24965	hypothetical protein	1.091995	-5.7761	-5.7761	7.223623	0.000198	0.027965	0.76029
BUE60_21340	hypothetical protein	0.489961	-0.87607	-6.23156	7.435516	0.000214	0.029594	0.678285
BUE60_01650	thiosulfate sulfurtransferase	-6.07151	0.083998	-6.07151	6.817511	0.000234	0.031502	0.867404
BUE60_10700	molecular chaperone DnaK	0	6.590735	7.732228	7.78859	0.000254	0.033474	0.499649
BUE60_27730	glycoside hydrolase 68 family protein	1.990119	1.550896	1.567534	10.9663	0.000266	0.034293	0.908225
BUE60_02230	prevent-host-death protein	0.759926	-5.94885	-5.94885	7.190749	0.000284	0.035773	0.772524
BUE60_17400	transposase	-6.7498	-0.97421	-6.7498	7.043704	0.0004	0.049323	0.710718
BUE60_09700	30S ribosomal protein S3	-0.25804	-0.86626	-1.83476	10.5992	0.000433	0.052335	0.756287
BUE60_25785	chaperonin GroL	0.57601	-1.49145	-0.64014	10.64873	0.000447	0.052893	0.806498
BUE60_23685	type III chaperone protein ShcN	0.791859	2.274048	1.261047	10.05967	0.0005	0.057302	0.729401
BUE60_06660	hypothetical protein	0.308284	0.295896	-5.96605	7.341313	0.000504	0.057302	0.772319
BUE60_12330	cysteine hydrolase	2.283692	2.685102	-4.08027	7.224962	0.000553	0.060751	0.598509

BUE60_18905	HslU--HslV peptidase proteolytic subunit	1.65815	0.192043	2.487488	9.847487	0.000566	0.060751	0.826576
BUE60_28885	IS66 family transposase	0.407687	-6.25623	-6.25623	7.261808	0.000566	0.060751	0.430101
BUE60_26610	transposase	0	7.163183	8.603967	8.477509	0.000609	0.064132	0.24148
BUE60_19335	hypothetical protein	3.39819	1.071081	-4.22353	7.607979	0.000658	0.065776	0.780608
BUE60_17470	1-(5-phosphoribosyl)-5-((5-phosphoribosylamino) methylideneamino)imidazole-4-carboxamide isomerase	0.1607	-6.29092	-6.29092	7.188882	0.000652	0.065776	0.404107
BUE60_12825	GntR family transcriptional regulator	-3.71429	-7.56884	-3.23523	7.521741	0.000643	0.065776	0.622523
BUE60_29200	hypothetical protein	-2.68519	-7.0603	-7.0603	7.110864	0.000695	0.068254	0.751303
BUE60_07150	IS3 family transposase	3.570854	-3.93558	-3.93558	7.380453	0.000707	0.068298	0.528357
BUE60_21075	phna protein alkylphosphonate uptake	0.018146	-1.62145	-6.47975	7.385461	0.000726	0.068957	0.759865
BUE60_27745	hypothetical protein	1.89857	0.396512	-6.92905	8.922955	0.000745	0.069619	0.644543
BUE60_21475	ABC transporter substrate-binding protein	2.601267	0.065625	0.341459	8.703373	0.000796	0.073259	0.828218
BUE60_28550	hypothetical protein	6.282644	8.43938	0	7.912608	0.000845	0.075941	0.40412
BUE60_15495	hypothetical protein	1.808798	2.130559	1.632545	9.785202	0.000852	0.075941	0.825144
BUE60_22845	hypothetical protein	-6.87515	1.564223	-6.87515	8.061091	0.000944	0.082881	0.365741
BUE60_02805	D-hexose-6-phosphate mutarotase	-0.98576	-3.05392	-1.17007	9.230321	0.000979	0.084697	0.784073
BUE60_28540	phospholipase	0	7.511676	0	6.884314	0.001001	0.085306	0.45
BUE60_15770	dipeptide ABC transporter permease DppC	-3.81395	3.113897	-3.81395	6.646059	0.001023	0.085963	0.866672
BUE60_25850	IS630 family transposase	-5.25668	2.859024	-5.25668	7.527171	0.001048	0.08674	0.421919
BUE60_23415	hypothetical protein	-6.49249	-0.63362	-6.49249	6.929545	0.001133	0.092485	0.629163
BUE60_06270	efflux transporter periplasmic adaptor subunit	-1.16271	-0.59189	-6.46943	7.196219	0.00117	0.092932	0.674268
BUE60_02790	sugar ABC transporter permease	-1.60463	-1.04277	-1.66657	10.4278	0.001167	0.092932	0.729736
BUE60_11475	Cd(II)/Pb(II)-responsive transcriptional regulator	1.789462	1.420796	-4.80667	7.242217	0.001252	0.098092	0.730896
BUE60_08690	flagellar biosynthetic protein FliO	1.326382	-5.15132	-5.15132	6.890662	0.001273	0.098371	0.849329
BUE60_28090	transposase	0	7.452437	0	6.844453	0.001307	0.098371	0.45
BUE60_19630	hypothetical protein	-2.56629	-6.85827	-6.85827	6.97748	0.001307	0.098371	0.925232
BUE60_01880	phage tail protein	6.780487	5.307834	6.57077	7.564627	0.001339	0.099517	0.635214

3   [a]Gene expression ratio in 27ºC-HSC medium against 27ºC-HS medium.

4   [b]Gene expression ratio in 18ºC-HS medium against 27ºC-HS medium.

5   [c]Gene expression ratio in 18ºC-HSC medium against 27ºC-HS medium.

Expression of *cfa1* and *cmaD* of Psa6 and Psg by RT-qPCR analysis

1 **Table 3. Expression of *cfa1* and *cmaD* of Psa6 and Psg by RT-qPCR analysis**

Gene	Condition	0h	3h	6h	Gene	Condition	0h	3h	6h
Psa6 *cfa1*	27HS	0.00	**1.87**	**2.83**	Psg *cfa1*	27HS	0.00	0.08	**3.84**
	27HSC	0.00	0.79	**2.43**		27HSC	0.00	0.27	**3.76**
	27LB	0.00	-3.90	-4.55		27LB	0.00	0.58	**3.73**
	27*hrp*	0.00	-3.46	-3.88		27*hrp*	0.00	0.29	**3.55**
Psa6 *cmaD*	27HS	0.00	**3.72**	**5.73**	Psg *cmaD*	27HS	0.00	0.18	-0.29
	27HSC	0.00	**1.32**	**5.41**		27HSC	0.00	0.57	0.06
	27LB	0.00	0.89	**1.30**		27LB	0.00	0.86	-0.58
	27*hrp*	0.00	0.78	**1.40**		27*hrp*	0.00	0.57	-0.56

2 The expression level (Log_2) is indicated as a ratio relatively against 0h of each culture condition.

3 Bold letters indicate that expression is induced ($Log_2 > 1.0$), and blue letters indicate that

4 expression is suppressed ($Log_2 < -1.0$).

Expression of *argK* and *argD* of Psa6 and Psa1 by RT-qPCR analysis

1 **Table 4. Expression of *argK* and *argD* of Psa6 and Psa1 by RT-qPCR analysis**

Gene	Sample	0h	3h	6h	Gene	Sample	0h	3h	6h
Psa6 *argK*	27HS	0.00	**4.81**	**3.13**	Psa1 *argK*	27HS	0.00	0.28	**1.78**
	27HSC	0.00	**4.93**	**2.66**		27HSC	0.00	-0.24	0.89
	27LB	0.00	**1.85**	**3.78**		27LB	0.00	**2.09**	0.85
	27*hrp*	0.00	**1.31**	**2.92**		27*hrp*	0.00	**1.97**	0.95
Psa6 *argD*	27HS	0.00	0.89	**2.74**	Psa1 *argD*	27HS	0.00	-0.30	0.12
	27HSC	0.00	0.91	**2.04**		27HSC	0.00	-0.71	-0.35
	27LB	0.00	-5.12	-5.20		27LB	0.00	-0.01	0.30
	27*hrp*	0.00	-5.39	-5.29		27*hrp*	0.00	-0.01	0.03

2 The expression level (Log_2) is indicated as a ratio relatively against 0h of each culture condition.

3 Bold letters indicate that expression is induced ($Log_2 > 1.0$), and blue letters indicate that

4 expression is suppressed ($Log_2 < -1.0$).

Figure 1

Comparison of expression trends in the RNA-seq and RT-qPCR analyses.

The expression levels of the virulence genes in RNA-Seq were plotted on the vertical axis and that in RT-qPCR were plotted on the horizontal axis. Most of the plots were concentrated in the first and third quadrants, while some were concentrated in the second and fourth quadrants . The yellow wavy line indicates a linear approximation, and R^2 value (about 0.6) was obtained.
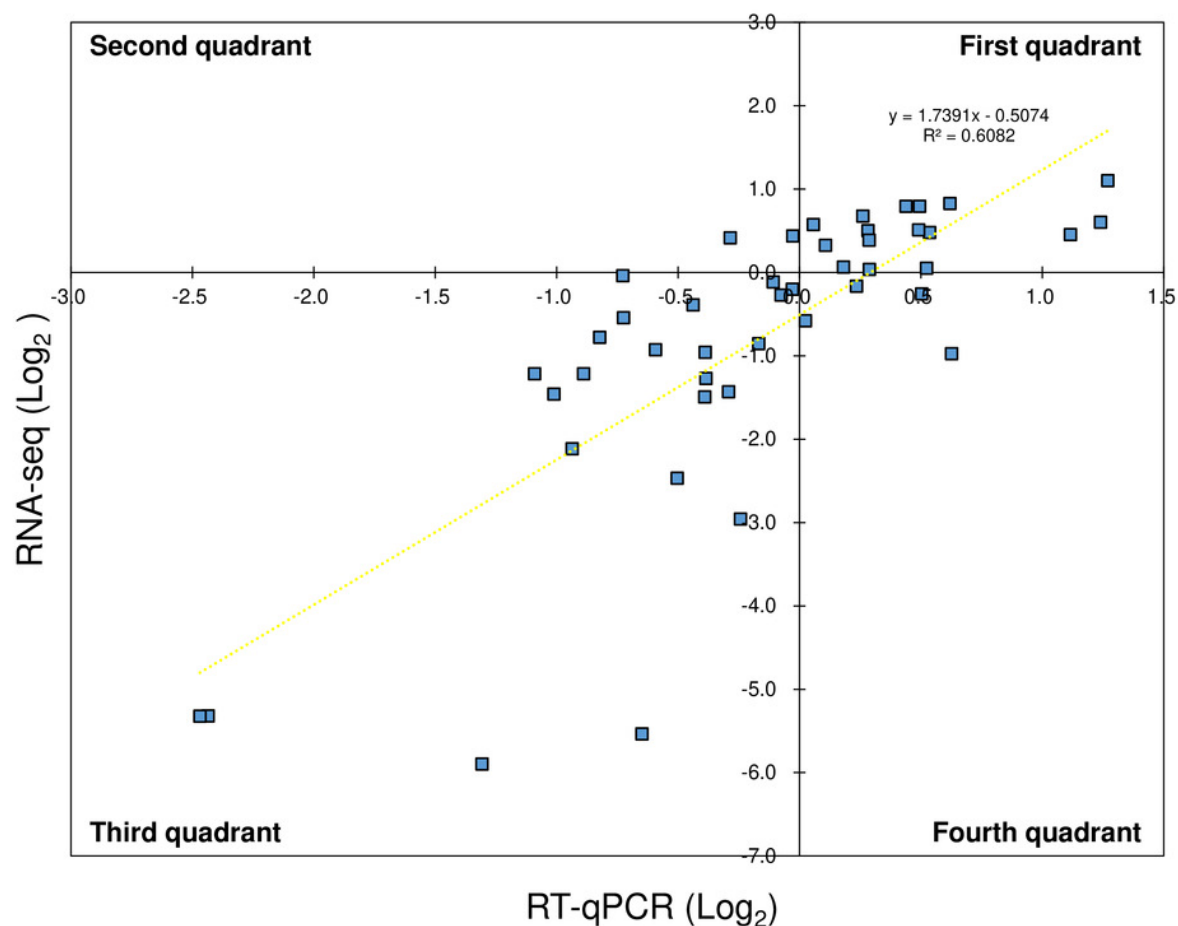
Figure 2

Heat map constructed from the expression level of virulence genes of Psa6 under each culture condition.

The results of RT-qPCR analysis of Psa6 were clustered using the unweighted pair group method with arithmetic mean (UPGMA). **A**, Heat map of expression levels of virulence genes under each culture condition. **B**, Reconstructed heat map excluding the conditions of the *hrp*-inducing medium. Gene names highlighted in blue are phaseolotoxin synthesis genes, and gene names highlighted in yellow are coronatine synthesis genes. Remaining genes are type III effectors. In heat maps, induced genes are indicated as yellow boxes and suppressed genes are indicated as blue boxes.
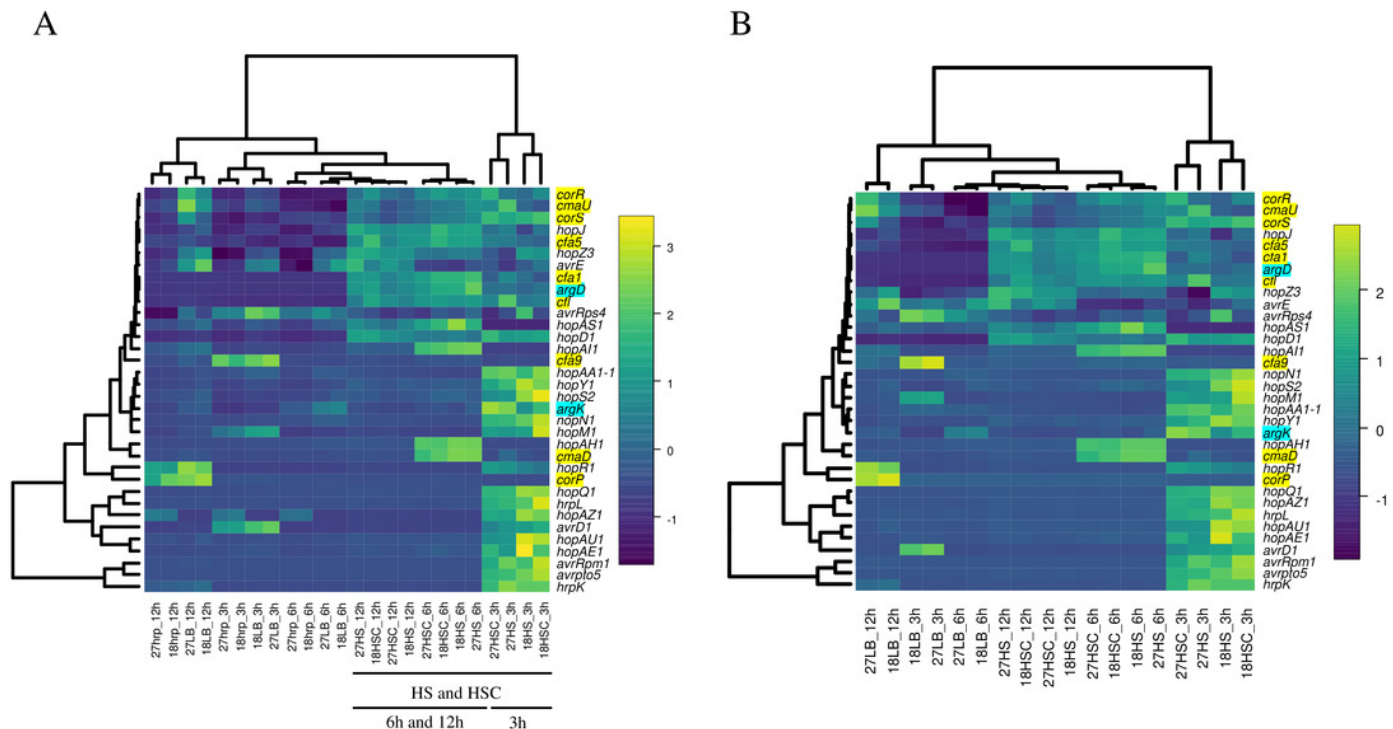
Figure 3

Heat map constructed from the expression levels of virulence genes of Psa6, Psa1, Psa3, and Psg under each culture condition.

The results of RT-qPCR analysis of Psa6, Psa1, Psa3, and Psg were clustered using the unweighted pair group method with arithmetic mean (UPGMA). **A**, Heat map of expression levels of virulence genes under each culture condition. **B**, Reconstructed heat map excluding the results of both Psa6 and Psg. In heat maps, induced genes are indicated as yellow boxes and suppressed genes are indicated as blue boxes.
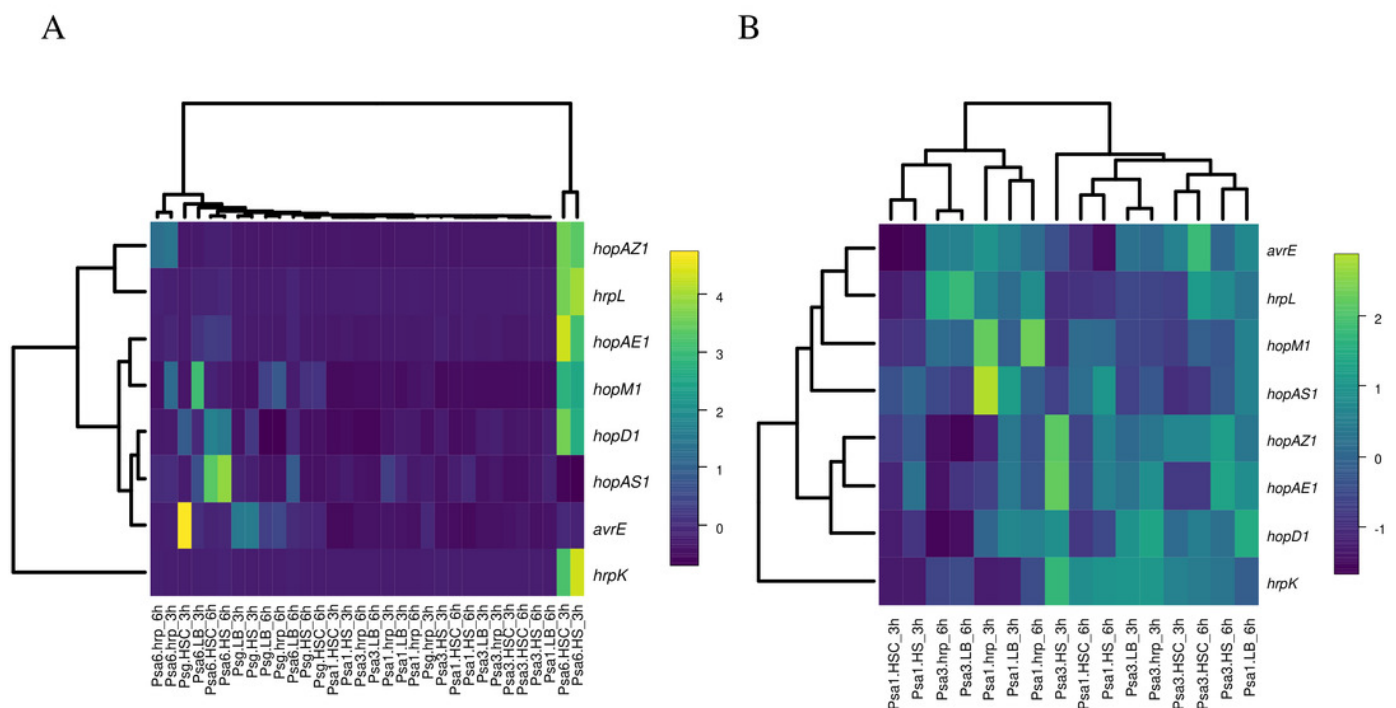
Figure 4

Schematic diagram of the expression pattern of Psa6 virulence genes in different media.

The expression of virulence genes (type III effector genes and phytotoxin synthesis genes) of Psa6 was induced in the coronatine-producing media, and the expression behavior had two patterns: early-inducible and late-inducible. These genes were suppressed or constant in LB medium and the *hrp*-inducing medium.