Gene Expression of Type VI Secretion System Associated with Environmental Survival in *Acidovorax avenae* subsp. *avenae* by Principle Component Analysis

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Abstract: Valine glycine repeat G (VgrG) proteins are regarded as one of two effectors of Type VI secretion system (T6SS) which is a complex multi-component secretion system. In this study, potential biological roles of T6SS structural and VgrG genes in a rice bacterial pathogen, *Acidovorax avenae* subsp. *avenae* (Aaa) RS-1, were evaluated under seven stress conditions using principle component analysis of gene expression. The results showed that growth of the pathogen was reduced by H$_2$O$_2$ and paraquat-induced oxidative stress, high salt, low temperature, and vgrG mutation, compared to the control. However, pathogen growth was unaffected by co-culture with a rice rhizobacterium *Burkholderia seminalis* R456. In addition, expression of 14 T6SS structural and eight vgrG
genes was significantly changed under seven conditions. Among different stress conditions, high salt, and low temperature showed a higher effect on the expression of T6SS gene compared with host infection and other environmental conditions. As a first report, this study revealed an association of T6SS gene expression of the pathogen with the host infection, gene mutation, and some common environmental stresses. The results of this research can increase understanding of the biological function of T6SS in this economically-important pathogen of rice.

**Keywords:** T6SS gene expression; gene knockout; co-culture; *in vivo*; stress; principle component analysis

1. Introduction

Secretion of proteins through secretion systems is a way by which bacteria influence their extracellular surroundings and other bacteria [1]. In pathogenic bacteria, the secretion systems which transfer proteins and toxins into the environment and within a eukaryotic target cell are important for their virulence and survival in hosts [2,3]. At least six distinct multi-component secretion systems (referred to type I–VI secretion system, or T1SS–T6SS) are used by Gram-negative bacterial pathogens to transport the proteins across the membranes of the bacteria and, eventually, the host [4–8]. The T6SS is a newly found multi-component secretion system, which is often involved in interaction with eukaryotic hosts in either pathogenic or symbiotic relationships [9]. According to previous studies, most of T6SS-containing bacteria are known as human and animal pathogens [7,10]. The T6SS encoded by clusters of contiguous genes is composed of 13 conserved proteins and a variable complement of accessory elements. It is reported that they are present in one or more copies in numerous Gram-negative bacterial pathogens including *Vibrio cholerae, Pseudomonas aeruginosa, Yersinia pestis, Escherichia coli, Salmonella enterica, Agrobacterium tumefaciens, Rhizobium leguminosarum, Francisellatularensis, Burkholderia mallei, and Edwardsiella* spp. In addition, the T6SS genes have key roles in virulence-related processes in some of these bacterial pathogens [11–18].

In bacterial pathogens, infection of the host depends on effective colonization, as well as survival, of the pathogen in the host and environment by resistance against different stress conditions [19–21]. Researchers have shown that T6SS plays an important role in pathogens to show resistance to environmental stresses [22–24]. For example, *pppA-ppkA* null mutant decreased resistance to H$_2$O$_2$-induced oxidative stress of *P. aeruginosa* [25]. Weber *et al.* (2009) reported a new role for T6SS in the ecology of *Vibrio anguillarum* and attributed this role to a signal-sensing mechanism that modulates expression of regulators of the general stress response [26]. These results indicate that there is a potential interaction between stress conditions and T6SS genes in bacterial pathogens of human and animals.

Recently, T6SSs of plant pathogens such as *Pseudomonas syringae* and *Pectobacterium wasabiae* have been studied [27–29]. Furthermore, genome-wide analysis revealed the existence of only one T6SS gene cluster in strain RS-1 of *Acidovorax avenae* subsp. *avenae* (Aaa), a widely-distributed seed-borne pathogen of rice [30]. In addition, our previous study showed that the homolog of VgrG, as one of two most important putative T6SS effectors, cannot be encoded by a T6SS cluster but it is considered as an orphan
component in Aaa RS-1 [31]. The objective of this study was to assess the potential interaction between expression of T6SS structure and VgrG genes of Aaa RS-1 and common environmental stress conditions, including in vivo infection, co-culture with rice rhizobacterium Burkholderia seminalis R456, and one vgrG (Aave_0497) mutation, as well as high salt, low-temperature, H$_2$O$_2$- and paraquat-induced stresses.

2. Results

2.1. Repression of Bacterial Growth under Environmental Stress Conditions

The growth of Aaa strain RS-1 was significantly inhibited by high salt, low temperature, H$_2$O$_2$- and paraquat-mediated oxidative stress compared to the corresponding control. The survival of Aaa RS-1 under conditions of high salt, low temperature, H$_2$O$_2$ and paraquat stress is shown in Figure 1. Results from this study indicated that nutrient broth (NB) supplemented with 2.0% and 3.0% NaCl caused a reduction for 66.1% and 83.1%, respectively, in the growth of Aaa RS-1 compared to the control (1.0% NaCl). No statistical difference was observed among the concentrations of NaCl higher than 3.0% (Figure 1a). Compared to 30 °C (control), low temperature at 15 °C caused a 27.1%, 71.2%, and 75.9% reduction in the growth of Aaa RS-1 after 6, 12, and 24 h, respectively (Figure 1b). Furthermore, H$_2$O$_2$ at 8, 16, and 32 mM caused a significant reduction of 73.0%, 98.2%, and 98.2%, respectively, in the growth of Aaa RS-1 (Figure 1c). Paraquat at 10, 50, and 100 µM caused a 16.0%, 58.0%, and 61.8% reduction, respectively, in the growth of Aaa RS-1 after 24 h compared to the control. The growth of Aaa RS-1 was unaffected by 10 µM of paraquat, but was significantly reduced by 50 and 100 µM of paraquat (Figure 1d). In addition, NB supplemented with 2.0% NaCl, culture at 15 °C, NB supplemented with 8 mM H$_2$O$_2$ and NB supplemented with 50 µM paraquat for 24 h were selected as repression points for salt-induced osmotic stress, low temperature stress, H$_2$O$_2$-induced oxidized stress, and paraquat-induced oxidized stress, respectively.

2.2. Repression of Bacterial Growth during in Vivo Infection and Co-Culture Condition

We did not count the number of bacteria during in vivo infection and did not compare with the number of bacteria cultured under in vitro condition because the bacterial density is obviously higher in the broth in vitro than in the plants in vivo. Furthermore, the growth of Aaa strain RS-1 was unaffected by the co-culture of bacteria with B. seminalis R456. The OD$_{600}$ value increased from 0.2 to 1.4 when bacteria were incubated in NB alone, while the OD$_{600}$ value increased from 0.2 to 1.1 after 24 h at 30 °C when bacteria were co-cultured in NB with B. seminalis R456.

2.3. Repression of Bacterial Growth in ΔvgrG-2

There was a significant difference in the growth between the wild type and the ΔvgrG-2 of Aaa strain RS-1. The OD$_{600}$ of wild type increased from 0.089 to 0.545, 0.910, and 1.348 after 12, 24, and 48 h of incubation at 30 °C, respectively. However, the OD$_{600}$ of ΔvgrG-2 was significantly inhibited by 50.6%, 57.1%, and 52.7% compared to the wild type after 12, 24, and 48 h of incubation at 30 °C, respectively. There was no significant difference in the growth between the wild type and complementary strain ΔvgrG-2(vgrG-2). In addition, there was no significant difference in the growth between the mutant strain and mock strain ΔvgrG-2(pRADK) (Figure 2).
Figure 1. Growth of *Acidovorax avenae* subsp. *avenae* RS-1 under different conditions of (a) NaCl-induced osmotic stress; (b) low temperature stress; (c) H$_2$O$_2$-mediated oxidative stress; (d) paraquat-mediated oxidative stress ($p < 0.05$). *Aaa* RS-1 incubated in NA broth with 1.0% NaCl at 30 °C, 200 rpm for 24 h was used as the negative control. Data from the repeated experiment were pooled and subjected to analysis of variance. Columns with the same letters (a–e) are not significantly different ($p = 0.05$). Error bars represent the standard error of the mean.

2.4. No Significant Difference for in Vitro Expression of T6SS Gene

Gene expression data obtained from quantitative real-time PCR (qPCR) showed that the Δ$C_t$ values of 22 T6SS genes (14 structural genes and eight vgrG genes) in NB (control) samples varied from 16.4 to 30.2 (Δ$C_t$) (Figure 3). This suggested that there was a slight difference in the gene expression of each T6SS component under *in vitro* condition. However, in general, this result revealed that the expression level of 22 different T6SS genes under *in vitro* condition was similar for this change is less than two-fold, which have been regarded as the standard to differentiate significant change from insignificant change.
Figure 2. Comparison of growth between the wild type and ΔvgrG-2 mutant of Acidovorax avenae subsp. avenae strain RS-1. WT: wild type strain; ΔvgrG-2: vgrG-2 mutant strain; ΔvgrG-2(vgrG-2): vgrG-2 complementary strain; ΔvgrG-2(pRADK): mock strain with empty pRADK plasmid. Data from the repeated experiment were pooled and subjected to analysis of variance. Error bars represent the standard error of the mean.

Figure 3. T6SS gene expression compared to 16S RNA gene, using quantitative real-time PCR in Acidovorax avenae subsp. avenae RS-1 under in vitro condition.
2.5. Different Expression of T6SS Gene under Various Environmental Conditions

Differential changes were observed in T6SS gene expression of Aaa strain RS-1 during in vivo rice infection, co-culture with B. seminalis R456, and vgrG-2 mutation as well as high salt, low-temperature, H\textsubscript{2}O\textsubscript{2}- and paraquat-mediated oxidative stress. The expression of 22 T6SS genes was dependent on both the kind of gene and the type of condition (Table 1). Compared to the corresponding control, it was obvious that salt stress and low temperature up-regulated expression of most genes. On the contrary, the expression of most genes was down-regulated during in vivo infection and in H\textsubscript{2}O\textsubscript{2}- and paraquat-induced oxidized stress. In addition, none of the 22 T6SS genes was up-regulated in the vgrG-2 mutant.

**Table 1.** Gene expression of T6SS using quantitative real-time PCR in Acidovorax avenae subsp. avenae RS-1 during in vivo rice infection, vgrG-2 mutant, and co-culture with rice rhizobacterium Burkholderia seminalis R456, as well as high salt, low temperature, H\textsubscript{2}O\textsubscript{2}- and paraquat-mediated oxidative stress.

| T6SS Gene | Expression Change Relative to that of in Vitro under the Conditions of | Salt * | Temperature | H\textsubscript{2}O\textsubscript{2} | Co-Culture | in Vivo | Mutant |
|-----------|------------------------------------------------|-------|------------|-------------|------------|--------|--------|
| clpB      | ↑1.3 ↑2.8 * ↓14.8 * ↓4.5 * ↑15.0 * ↓4.4 * ↓1140.8 * | ↓12.3 * ↑36.1 * ↓5.1 * ↓1.8 * ↓1.8 * ↓3.1 * ↓14.1 * |       |           |             |        |        |
| impA      | ↑120.9 ↑2.6 * ↓1.1 ↑4.4 * ↑7.4 * ↑2.6 * ↓27.0 * | ↑20.9 * ↑2.6 * ↓1.1 * ↑4.4 * ↑7.4 * ↑2.6 * ↓27.0 * |       |           |             |        |        |
| impB      | ↓1.4 * ↓1.5 * ↓7.9 * ↓10.2 * ↑1.6 * ↓2.2 * ↓3008.4 * | ↓40.1 * ↓1.5 * ↓1.1 * ↓1.8 * ↓1.8 * ↓3.1 * ↓14.1 * |       |           |             |        |        |
| duf877    | ↑2.0 * ↑3.8 * ↓4.5 * ↓2.0 * ↑1.4 * ↑1.9 * ↑28.8 * | ↓10.9 * ↑3.8 * ↓4.5 * ↓2.0 * ↑1.4 * ↑1.9 * ↑28.8 * |       |           |             |        |        |
| impF      | ↑23.2 * ↑63.5 * ↑9.5 * ↑1.5 * ↑3.4 * ↑4.2 * ↓11.8 * | ↑23.2 * ↑63.5 * ↑9.5 * ↑1.5 * ↑3.4 * ↑4.2 * ↓11.8 * |       |           |             |        |        |
| impH      | ↑30.2 * ↑1.2 * ↓1.0 * ↓1.0 * ↓1.0 * ↓3.1 * ↓13.4 * | ↑30.2 * ↑1.2 * ↓1.0 * ↓1.0 * ↓1.0 * ↓3.1 * ↓13.4 * |       |           |             |        |        |
| pppA      | ↑5.1 * ↑2.7 * ↓8.3 * ↓1.7 * ↓1.7 * ↓1.7 * ↓1.7 * | ↑5.1 * ↑2.7 * ↓8.3 * ↓1.7 * ↓1.7 * ↓1.7 * ↓1.7 * |       |           |             |        |        |
| lip       | ↑5.4 * ↓2.0 * ↓5.9 * ↓11.9 * ↓5.7 * ↓5.7 * ↓31.6 * | ↑5.4 * ↓2.0 * ↓5.9 * ↓11.9 * ↓5.7 * ↓5.7 * ↓31.6 * |       |           |             |        |        |
| impJ      | ↑51.2 * ↑70.9 * ↓1.6 * ↓1.6 * ↓1.6 * ↓1.6 * ↓1.6 * | ↑51.2 * ↑70.9 * ↓1.6 * ↓1.6 * ↓1.6 * ↓1.6 * ↓1.6 * |       |           |             |        |        |
| dotU      | ↓6.4 * ↓16.9 * ↓15.4 * ↓37.6 * ↓1.7 * ↓10.9 * ↓10.9 * | ↓6.4 * ↓16.9 * ↓15.4 * ↓37.6 * ↓1.7 * ↓10.9 * ↓10.9 * |       |           |             |        |        |
| icmF      | ↑13.6 * ↑2.4 * ↑1.4 * ↑1.1 * ↑1.1 * ↑1.1 * ↑1.1 * | ↑13.6 * ↑2.4 * ↑1.4 * ↑1.1 * ↑1.1 * ↑1.1 * ↑1.1 * |       |           |             |        |        |
| impM      | ↓2.4 * ↓2.5 * ↓6.2 * ↓2.2 * ↓2.2 * ↓2.2 * ↓2.2 * | ↓2.4 * ↓2.5 * ↓6.2 * ↓2.2 * ↓2.2 * ↓2.2 * ↓2.2 * |       |           |             |        |        |
| duf1305   | ↑39.5 * ↑115.5 * ↓1.1 * ↓1.1 * ↓1.1 * ↓1.1 * ↓1.1 * | ↑39.5 * ↑115.5 * ↓1.1 * ↓1.1 * ↓1.1 * ↓1.1 * ↓1.1 * |       |           |             |        |        |
| vgrG-1    | ↑1.0 ↓1.2 ↓1.4 ↓2.2 * ↑1.7 * ↑1.0 ↓1.8 |       |           |             |        |        |
| vgrG-2    | ↑1.1 ↑1.7 ↓1.8 ↑1.1 * ↑2.4 * ↓2.4 * ↓2.1 * |       |           |             |        |        |
| vgrG-3    | ↓4.0 * ↑1.6 ↓3.0 * ↓4.4 * ↓2.1 * ↓2.1 * ↓2.1 * |       |           |             |        |        |
| vgrG-4    | ↑14.8 * ↑17.1 * ↑3.1 * ↑3.1 * ↑3.1 * ↑3.1 * ↑3.1 * |       |           |             |        |        |
| vgrG-5    | ↑20.6 * ↑597.0 * ↑1028.9 * ↑77.4 * ↑20.1 * ↑108.3 * ↑123.9 * |       |           |             |        |        |
| vgrG-6    | ↑1.1 ↓1.8 ↓1.45 * ↓7.0 * ↓6.5 * ↓2.2 * ↓2.2 * |       |           |             |        |        |
| vgrG-7    | ↑1.6 ↓1.4 ↑2.8 * ↓1.5 ↓1.5 * ↓1.5 * ↓1.5 * |       |           |             |        |        |
| vgrG-8    | ↓1.9 ↓4.0 * ↓18.1 * ↓9.4 * ↓6.6 * ↓3.5 * ↓11.1 * |       |           |             |        |        |

* Salt: 2% NaCl high salt; Temperature: 15 °C low temperature; Co-culture: co-culture with rice rhizobacterium Burkholderia seminalis R456; Mutant: vgrG-2 gene knockout mutant; H\textsubscript{2}O\textsubscript{2}: 8 mM H\textsubscript{2}O\textsubscript{2}-mediated oxidative stress; Paraquat: 50 µM paraquat-mediated oxidative stress; In vivo: in vivo rice infection. ↑: up-regulation; ↓: down-regulation; *: the change of gene expression is more than two fold compared to the corresponding in vitro control.
In this study, the transcript of almost all structural genes (except dotU) and three vgrGs (vgrG-2, vgrG-4, vgrG-6) showed their maximum repression level of expression in response to vgrG-2 mutant. Five structural genes (impB, impH, pppA, hiP and imcF) showed their maximum activation level of expression in response to in salt stress; six structural genes (impA, impE, impF, impJ, impM, duf1305) and one vgrG-4 showed their maximum activation level of expression under low temperature stress. Based on the results, only one structural gene clpB and one vgrG-2 showed the highest level of expression under co-culture condition; three vgrGs (vgrG-3, vgrG-5, vgrG-7) showed their maximum activation level of expression under paraquat stress; and two vgrGs (vgrG-1 and vgrG-3) showed their maximum repression level of expression under H2O2 stress. Interestingly, no gene showed its special expression levels during in vivo infection (Table 1).

Salt stress caused differential expression of 16 genes (13 structural genes and three vgrGs), including 12 up-regulated genes and four down-regulated genes. Low temperature caused differential expression of 15 genes (12 structural genes and three vgrGs), including 12 up-regulated genes and three down-regulated genes. H2O2 stress caused differential expression of 15 genes (10 structural genes and five vgrGs), including four up-regulated genes and 11 down-regulated genes. In addition, Paraquat stress caused differential expression of 15 genes (nine structural genes and six vgrGs), including five up-regulated genes and 10 down-regulated genes. Co-culture caused differential expression of 15 genes (nine structural genes and six vgrGs), including nine up-regulated genes and six down-regulated genes. In vivo infection caused differential expression of 13 genes (eight structural genes and five vgrGs), including five up-regulated genes and eight down-regulated genes. None of the 14 structural genes was significantly up-regulated in the vgrG-2 mutant, but found to be down-regulated.

2.6. Principle Component Analysis

The relative expression (RE) levels of 22 T6SS genes under six stress conditions (no vgrG mutant condition) were subjected to principle component analysis (PCA). The data for gene expression in mutant condition was excluded from analyses because of the oversize (incalculable) change in the expression levels of several genes. The first and second principal components of PCA plot (PC1 and PC2) accounted for 37.6%, and 24.5% of the variation, respectively, in the dataset (Figure 4). The contribution of conditions and genes as well as their interactions were determined based on the scores of PC1 and PC2. The relative importance of stress conditions and genes was determined based on their distance to the origin, the point where the two axes cross at zero on both scales. More distant (stress conditions and genes) from the origin was considered as more important factor for the expression levels.

The PCA results of condition factors in this study indicated that low temperature and high salt stresses were the most important contribution to the variation of RE levels, followed by H2O2, paraquat and co-culture stresses, while in vivo infection has the least contribution to the variation of RE levels based on their distance to the origin (Figure 4a). In addition, this study revealed three different types of contribution of stress types to the variation of RE levels. The different kinds of stress were grouped into three groups and significantly separated from left to right along the PC1 axis (p < 0.001) in order of group 1: (paraquat, H2O2, co-culture), group 2: (in vivo) and group 3: (temperature, salt).

Combining the PC1 axis and PC2 axis, the loadings of individual gene RE levels distributed on each quadrant indicated those genes are more related to these conditions that were on the same
quadrant. The 22 T6SS genes were distributed in four quadrants by PC1 and PC2 axis. Some individual genes, like *pppA*, *dotU* and *vgrG*-5 were noted. *pppA* was close to PC2 axis, which indicated that *pppA* may be not only interact with paraquat and co-culture conditions but also with temperature condition. Furthermore, *dotU* and *vgrG*-5 were close to origin, revealing that they may be able to, at least partially, interact with almost all conditions (Figure 4b). However, *dotU* and *vgrG*-5 have the least contribution to the RE levels based on the distance to the origin.

**Figure 4.** Two-dimensional principle component analyses (PCA) of 14 T6SS genes expression of *Acidovorax avenae* subsp. *avenae* RS-1 under 6 different conditions. (a) Scores from six different conditions; (b) loadings of the individual gene from the PCA of the relative expression data. S: 2% NaCl high salt; T: 15 °C low temperature; H: 8 mM H₂O₂-mediated oxidative stress; P: 50 µM paraquat-mediated oxidative stress; C: Co-culture with rice rhizobacterium *Burkholderia seminalis* R456; V: *In vivo* rice infection. The *vgrG*-2 mutation was excluded in PCA for the oversize change in several T6SS genes expressions. The different kinds of conditions were grouped into two groups and significantly separated from left to right along the PC1 axis in the order of (paraquat, H₂O₂, co-culture), (*in vivo*) and (temperature, salt) (*p* < 0.001).

### 3. Discussion

Bacterial pathogens are often exposed to various kinds of environmental stresses during host infection. Furthermore, a particular bacterial species is likely to encounter numerous taxonomically-different
competitors in host and natural ecosystems [32]. Therefore, the molecular survival mechanism of bacteria is mainly dependent on their adaptation to the different hosts and environmental conditions. Identification of specific genes and gene expression patterns is important for studying the host infection and natural environmental survival of a bacterial pathogen. Recently, a review of T6SS indicated that T6SS may play key roles in microbial communities leading to more contributions to microbial interactions for environmental benefits [33]. Although T6SS is involved in a series of bacterial process, no research has been carried out to assess the effect of T6SS and its expression to host infection and environmental stresses. On the other hand, VgrGs, as one of the two putative effectors of T6SSs, are considered to play an important role in the function of T6SS machine [34–37]. Therefore, ecological roles of T6SS in Aaa RS-1 could be, at least in part, determined by understanding the effect of host infection, natural environmental stresses and gene mutation on the expression pattern of T6SS structural genes and vgrGs.

In agreement with previous research, this study revealed that bacteria adapt to different conditions such as low temperature, osmotic, and oxidative-induced stresses by changing the growth rate. It is known that bacteria are often subject to these stresses in the natural environment [19,38,39]. However, the extent of the changes depends on the type of conditions. T6SS was also found to be able to respond to a variety of environmental conditions. In this study, four common environmental stress conditions were chosen to get the stress repression point for the growth of Aaa RS-1 during in vivo infection, co-culture with B. seminalis R456 (a rice rhizobacterium) and vgrG-2 mutation, to evaluate the efficacy of T6SS gene expression in adaptation to these seven stress conditions. Although a broad set of differentially-expressed genes have been observed in the response of bacteria to osmotic stress [25,40], no research was found about the role of T6SS in bacterial response to the salt stress. However, in this study, the result of PCA indicated that salt stress was one of the most important stress effectors interacted with T6SS gene expression. Four T6SS structure genes including icmF, impB, impH, and duf877 were tightly interacted with salt-induced osmotic stress in PCA suggesting that the formation of T6SS structure may be related with the response of Aaa RS-1 to salt stress. Furthermore, little information is available about the role of VgrGs in bacterial response to environmental stresses. In this study, it became clear that the expression of most vgrGs (except vgrG-4) is affected by paraquat stress, H2O2 stress, and co-culture condition. However, the expression level of some vgrGs was not equal in paraquat stress and co-culture conditions, which suggest that multiple VgrGs in Aaa RS-1 maybe not be the result of a duplication but rather a gain of specific function, such as complementary expression in stress conditions. Although paraquat and H2O2 have a similar induced-oxidase mechanism, they were placed in the same group, but different spatial compartment, of PCA, indicating the complexity of the interaction between T6SS especially VgrGs with paraquat and H2O2.

T6SS has been experimentally shown to play a role in virulence in many cases. It may have an effect on limiting bacterial replication or virulence, increasing interaction with hosts other than pathogenesis which can lead to commensal or mutualistic conditions [41]. In some cases, putative T6SS components contribute to virulence, but in a manner that appears to be independent of other T6SS components [41]. Furthermore, expression of T6SS gene has been initially discovered as being specifically induced in vivo in many animal and human pathogens [42,43]. In this study, in vivo expressions of more than 50% of T6SS genes (13 of 22) were significantly changed, which showed that the T6SS genes were involved in the interaction between Aaa RS-1 and host. However, in contrast
with the up-regulation in other animal and human pathogens [42,43], expression of most genes was down-regulated among 13 genes in Aaa RS-1, suggesting the complexity of T6SS expression in plant pathogenic bacteria. On the other hand, the transcriptomic level of gene expression may be not always having a positive correlation with translation level of protein. Several studies also revealed that T6SS, in most cases, are not critical factors of pathogenesis, but rather improve the efficiency of colonization and/or infection of bacterial pathogens by attacking or killing the other bacteria [44]. Similar to the result of previous studies [44], this study also revealed that the expression of 15 genes was altered in Aaa RS-1 when confronted with rice rhizospheric bacterium B. Seminalis. It may indicate that T6SSs can, at least in part, contribute to the interaction of Aaa RS-1 with other bacteria.

The result of PCA indicated that two genes including dotU and vgrG-5 were very close but near to the origin point. This suggests that the two genes may be able to interact with all conditions such as in vivo infection condition. Interestingly, the in vivo expression change of dotU (OmpA/MotB) was more than 10-fold compared to the control (in vitro), suggesting that dotU has stronger response to host infection than other T6SS genes. Furthermore, in agreement with Broms et al. (2012) [37], the role of dotU in the virulence of Aaa RS-1 was justified by the fact that its mutant lost or significantly reduced virulence to rice plants (data not shown). In addition, our previous study revealed that OmpA/MotB domain containing proteins was in vivo expressed in Aaa RS-1 [32], while the interaction between DotU and IcmF has also been identified in Aaa RS-1 (data not shown). In this study, there was an indirect correlation between the expressions level of dotU and icmF in all six conditions of qPCR, indicating the negative-interaction between them.

PCA revealed a slight effect of vgrG-5 as it was close to the origin point. However, the expression of vgrG-5 was significantly changed in all conditions. The contrast result may be due to this fact that qPCR revealed differences in expression of T6SS gene under the same condition or one T6SS gene under seven conditions. In addition to vgrG-5, study of the other VgrG-coding genes is a good area of research because they encode the most important putative T6SS effectors. In this study, eight vgrGs which have high homology to each other in Aaa RS-1 were picked up for examination of gene expression. The result showed that the vgrGs were more sensitive to the stress group of (paraquat, H2O2, co-culture) than the stress group of (temperature, salt).

This study revealed the considerable changes of T6SS gene expression in Aaa RS-1 under in vivo infection, co-culture with rhizobacterium, and vgrG-2 mutant as well as high salt, low-temperature, H2O2- and paraquat-induced oxidative stresses. The extent of the changes depended on the type of conditions. The expression of the T6SS gene under these stress conditions was analyzed by qPCR. As a first study, PCA was used in exploring the potential interaction between T6SS gene expression and stress conditions. In general, the result of qPCR and PCA showed that high salt and low temperature had a higher impact on expression of T6SS structural genes whereas expression of vgrGs was more sensitive to H2O2- and paraquat-induced oxidative stresses. Expression of almost all T6SS structural genes was highly repressed in vgrG-2 mutant. Therefore, this study might provide a clue for further studies about the role of T6SS in the response of Aaa RS-1 to host infection, taxonomically different competitors, gene mutation, and various kinds of environmental stresses.
4. Experimental Section

4.1. Bacterial Strains, Plasmids and Chemicals

Bacteria and plasmids used in this study are listed in Table 2. *Aaa* RS-1 and *B. seminalis* R456 were isolated from diseased rice plants [45] and rice rhizosphere [46], respectively. Bacteria were stored in 20%–30% sterile glycerol (Shanglin Industries, Hangzhou, China) at −80 °C. Sodium chloride (NaCl) was obtained from Shisihewei Chemical Reagent Co., Ltd. (Shanghai, China), H₂O₂ was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and paraquat (paraquat dichloride X-hydrate) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacterial strains were regularly cultured and maintained at initial concentration of 10⁵ CFU/mL in NB (peptone 10.0 g/mL, yeast extract 3.0 g/mL, NaCl 5.0 g/mL, pH 7.5) at 30 °C, 200 rpm for 24 h, unless specifically described.

| Strain or Plasmid | Description | Source or Reference |
|-------------------|-------------|---------------------|
| Strains           |             |                     |
| Acidovorax avenae subsp. avenae |             |                     |
| RS-1              | The pathogen of bacterial brown stripe of rice, isolated from the diseased rice from Zhejiang province in China. Wild type strain in this study | Lab collection |
| ΔvgrG-2           | Km⁸; RS-1 in-frame deletion mutation defective in vgrG-2 | This study |
| ΔvgrG-2(pRADK)    | Km⁸; Chl⁹; complementary strain of ΔvgrG-2 complemented with pRADK-vgrG2 | This study |
| ΔvgrG-2(pRADK)    | Km⁸; Chl⁹; mock strain of ΔvgrG-2 with empty pRADK | This study |
| Burkholderia seminalis R456 | Isolated from rice rhizosphere from Zhejiang province in China. Biocontrol bacterium used in this study | Lab collection |
| Escherichia coli S17-1 λ pir | λ Lysogenic S17-1 derivative producing π protein for replication of plasmids carrying R6Kori; recAprohsdRRP4-2-Tc::Mu-Km::Tn7 λ pir | Liu et al. (2012) [47] |
| Plasmids          |             |                     |
| pJP5603           | Suicide vector; R6Kori, Km⁸ | Liu et al. (2012) [47] |
| pJP-G             | Km⁸; pJP5603 containing the 440 bp DNA fragment of gene vgrG-2 from Strain RS-1; used to create mutant ΔvgrG-2 | This study |
| pRADK             | Chl⁹; broad host expression vector | Liu et al. (2012) [47] |
| pRADK-vgrG2       | Chl⁹; pRADK plasmid containing the vgrG-2 gene and upstream fragment from strain RS-1, utilize to complement | This study |

Km⁸, Chl⁹: Kanamycin- and Chloromycetin-resistant, respectively.

4.2. Bacterial Growth in High Salt, Low Temperature and Oxidative Stress Conditions

In order to find the appropriate stress repression point for *Aaa* RS-1, the growth of the bacteria was firstly determined in different stress conditions of different concentrations. Bacterial adaptation to different environments was determined by examining cell growth in high salt, low temperature, and...
oxidative stress conditions. The 96-well microplates (Corning-Costar Corp., Corning, NY, USA) were used for this purpose. For salt stress treatment, each well in the 96-well microplates was inoculated with 200 µL of bacterial suspension (OD<sub>600</sub> = 0.1) with NaCl concentrations of 1.0% (Optimum concentration, served as the control), 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0%, 8.0%, and 9.0%. For low temperature treatment, bacterial growth was determined after incubating 200 µL of bacterial suspension (OD<sub>600</sub> = 0.1) at 15 and 30 °C (Optimum temperature, served as the control) for 0, 3, 6, 12 and 24 h, respectively. For H<sub>2</sub>O<sub>2</sub> treatment, bacterial suspension was incubated with 0.0 (the control), 8.0, 16.0, and 32.0 mM of H<sub>2</sub>O<sub>2</sub> at 30 °C for 24 h. The effect of paraquat on bacterial growth was determined by incubating the bacterial suspension (OD<sub>600</sub> = 0.3) with 0.0 (the control), 10.0, 50.0, and 100.0 µM of paraquat, at 30 °C for 0, 8, 16, and 24 h, respectively. The NB without bacteria was used as the negative control for this experiment. Finally, the OD<sub>600</sub> of each plate was determined and bacterial growth was evaluated based on the OD<sub>600</sub> of six biological replicates.

4.3. Bacterial Growth in Co-Culture and in Vivo Planta Conditions

The effect of co-culture on T6SS gene expression was examined by incubating Aaa RS-1 either alone or in combination with B. seminalis R456 according to Ruiz et al. (2009) [48]. Briefly, both bacterial strains were incubated in 40 mL of NB for overnight at 30 °C. In order to allow the interchange ofsecreted molecules present in the supernatants, the content of each tube was drawn in sterile syringe and a sterile filter of 32 mm diameter and 0.45 µm pore size (Pall–Newquay) was connected to each syringe. Both syringe–filter sets, each one containing one bacterial strain, were interconnected by means of single and sterile fused-silica tubing, and the media of both syringes were manually mixed every 2 h. Batch cultures for each strain were performed at the same time and the whole procedure was independently repeated at least twice.

The bacterial growth during in vivo rice infection was determined by collecting bacteria from diseased rice leaves directly as described in our previous study [32]. Briefly, bacterial strain was inoculated and recovered as follows. Leaves of four-week-old plants were infiltrated with sterilized syringe filled with 10 mL (~10<sup>8</sup> CFU/mL) of bacterial suspension. Six days after the inoculation, infected leaves were collected and decontaminated with alcohol. Leaves were cut into pieces with a sterile razor blade and maintained for 1 h in sterile glass plates containing 20 mL of distilled water. The incubation during this period of time allowed the bacteria to detach from the leaf tissues. The leaves were separated from the suspension and the bacterial cells were collected by centrifugation at 5000 RCF for 20 min. The bacterial cell pellets were then washed with phosphate buffer saline (PBS) and with water and then used for RNA extraction.

4.4. Construction of vgrG-2 Mutant and Complementation

The effect of gene mutation on bacterial growth and T6SS gene expression was determined by constructing one vgrG mutant of Aaa RS-1. In-frame deletion of vgrG-2 gene and complementation were performed as described of Liu et al. (2012) [47] by suicide plasmid pJP5603 through homologous recombination on the background of wild-type strain RS-1. For construction of the vgrG-2 deletion strain, 205 bp internal DNA fragment of vgrG-2 was PCR amplified with primers designed according to Aaa RS-1 genome. The PCR product was cloned into pMD19-T vector (TaKaRa, Dalian, China),
verified by sequencing and digestion with BamHI and EcoRI, and then ligated into the suicide vector pJP5603 to get pJP-G. For transfer of plasmid, the *E. coli* S17-1 λ pir has been employed and the resulting plasmid was then introduced into *Aaa* RS-1 via electroporation. This vgrG-2 gene may be targeted during transcription because of the high similarity between the internal fragments harbored by suicide plasmid and genomic DNA. The homologous recombination mutants were obtained after a single integrative recombination event, which disconnected essential protein domains, resulting in truncated and non-functional VgrG-2 protein. Mutant checking of the vgrG-2 among eight vgrGs of high sequence similarity were further confirmed by examining the expression of either side of the part knocked out using qPCR. In order to complement the ΔvgrG-2 strain, vgrG-2 open reading frame was amplified from the wide strain, along with 300 bp upstream of its start codon so that it included its native promoter. The 2652 bp PCR product was cloned into pGEM-T Easy vector, verified by sequencing, and then cloned into pRADK. The complementation vector was introduced into mutant cells by filter mating and selected by Chl + Km resistance. In addition, mock strain was constructed by introducing empty pRADK into mutant cells. The primers were listed in Table 3.

**Table 3.** Primers of T6SS genes used for quantitative real-time PCR (qPCR) of *Acidovorax avenae* subsp. *avenae* RS-1 in this study.

| T6SS Gene | Primer Sequence(5′→3′) | Target PCR Product of Function | Amplification Size (bp) |
|-----------|-------------------------|-------------------------------|------------------------|
| *clpB*    | F-GCAAGGGCGAGAAGGACAAG  | ATP-dependent chaperone ClpB   | 159                    |
|           | R-GCCGAGGAAACAGGAACGAG  |                               |                        |
| *impA*    | F-CTTGAACCTGCGGCGGACAC  | Type VI secretion-associated protein, ImpA family | 129                    |
|           | R-GCTCGGCCGGAATCACCAT   |                               |                        |
| *impB*    | F-ATCTCCCTCATCTGTCTCA   | Hypothetical protein Aave_2851 | 152                    |
|           | R-TCAGATCGGTCCCATCACAG  |                               |                        |
| *duf877*  | F-GCACCACCTGTCCACACACA  | Type VI secretion protein EvpB | 163                    |
|           | R-CGAACTGGCCGTATTTCTCT  |                               |                        |
| *impE*    | F-TGATCGGCTCGTCTGGCGGT  | Guanosine monophosphate reductase | 120                   |
|           | R-TGGTTGTAATACGCTCTGTTT  |                               |                        |
| *impF*    | F-TGGACTGGAAGGAGCTGGAA  | Type VI secretion system lysozyme-like protein | 126                    |
|           | R-AAGGGTGGTTGTGGTGTTGGA  |                               |                        |
| *impH*    | F-TGGAACTTCGGCCCTCTATGGG | Type VI secretion protein      | 121                    |
|           | R-TGGTGGGAAGATGTCCAGAGAA |                               |                        |
| *pppA*    | F-AGATCACGGGAGGACCATT   | Protein serine/threonine phosphatase | 214                   |
|           | R-TTCTCTGTCGTCGAGCAT    |                               |                        |
| *lip*     | F-GCAATGGCGATGGTGCCTCACTT  | Type VI secretion lipoprotein | 174                    |
|           | R-TCTGGGACCACGCTGATGCT   |                               |                        |
| *impJ*    | F-TCCAGAGATGCCACGGCAACA  | Type VI secretion protein, VC_A0114 family | 181                    |
|           | R-GAGCCAGGCTGGGAAATGAA  |                               |                        |
| *dotU*    | F-CCAGCATTACCTGCTCGAAAT  | DotU family type IV/VI secretion system protein | 196        |
|           | R-CCAGGTCTCGTGGTGTCGAGT  |                               |                        |
| *icmF*    | F-ACCCCGGCGGACCATCTCA   | Type VI secretion protein IcmF | 112                    |
|           | R-GCGAAGTCTACGCTCGTCA    |                               |                        |
Table 3. Cont.

| T6SS Gene | Primer Sequence (5’→3’) | Target PCR Product of Function | Amplification Size (bp) |
|-----------|--------------------------|--------------------------------|------------------------|
| impM      | F-GCAATGGCGTCGTCTCTC   | Adenylosuccinate synthase     | 192                    |
|           | R-CGGTGCGCTCCGGATCTTC   |                                |                        |
| duf1305   | F-GCCACAAGTTCCTTTTGC    | Type VI secretion protein, VC_0111 family | 202                    |
|           | R-AAAGACGGCCAGAATC      |                                |                        |
| vgrG-1    | F-ATCCGGTGGAAAAAGAATTCT | Rhs element Vgr protein Aave_0481 | 113                    |
|           | R-AAATAGATGCCCTTGTC     |                                |                        |
| vgrG-2    | F-GCGTCAATATGACGAGGC    | Rhs element Vgr protein Aave_0497 | 174                    |
|           | R-CCGCGGATAGAAGGAGAATC  |                                |                        |
| vgrG-3    | F-CGACAAGTGCCATACGAG   | Rhs element Vgr protein Aave_2047 | 121                    |
|           | R-TCGGTCTGTCGGACGCT     |                                |                        |
| vgrG-4    | F-CTGACCGGAGGCACGAGAT  | Rhs element Vgr protein Aave_2127 | 150                    |
|           | R-CCGAAACACCCACATCAACA |                                |                        |
| vgrG-5    | F-CATCAAGACGATTCCACG   | Rhs element Vgr protein Aave_2735 | 114                    |
|           | R-CAGCCATAATTGCTGTC     |                                |                        |
| vgrG-6    | F-ATACTGCGGTCAATATGAGC | Rhs element Vgr protein Aave_2840 | 185                    |
|           | R-GATTTTCCTCGGCGGATAG  |                                |                        |
| vgrG-7    | F-CCGATGGAAAAAGAATCAGC | Rhs element Vgr protein Aave_3347 | 111                    |
|           | R-AAATAGATGCCCTTGTC     |                                |                        |
| vgrG-8    | F-TCTTCCAGAAGTTCAGCC   | Rhs element Vgr protein Aave_0241 | 144                    |
|           | R-GGATTTTCGCGGAAGATT   |                                |                        |
| vgrG-2s   | F-TACCAGGGCGAAGATG     | Forepart fragment of the knockout fragment in vgrG-2 | 169                    |
|           | R-CGGGCCATCTCGGATAGTC  |                                |                        |
| vgrG-2b   | F-ACGGGGTGCTTCAAGATGG  | Tail fragment of the knockout fragment in vgrG-2 | 197                    |
|           | R-TGAGGGTGATGCTGTTT    |                                |                        |
| CvgrG-2   | F-ACACCACTTGCAGAAGTGCTG | vgrG-2 open reading frame with its promoter region | 2652                   |
|           | R-TGAGAGTGCGATTGCTTGC  |                                |                        |
| 16s RNA   | F-TTGGCGTGCCCTGCTTTCAT | Reference gene used for qPCR in this study | 120                    |
|           | R-CGGTAACAGGTCTCGGATGCT |                                |                        |

4.5. RNA Extraction and Gene Expression Analysis Using Quantitative Real-Time PCR

This experiment was conducted to assess the expression pattern of T6SS gene in Aaa RS-1 cells subjected to different stress treatments including: incubation in NB with 2.0% of NaCl for high salt stress, incubation at 15 °C for low temperature stress, incubation in NB with 8.0 mM of H2O2 and incubation in NB with 50.0 μM of paraquat for oxidative stresses, co-culture with B. seminalis R456 and finally inoculated and recovered from the host plant. Bacteria incubated in NB with 1.0% of NaCl at 30 °C were used as the control. All the in vitro treatments were cultured for 24 h. Total RNAs from each sample was extracted by using high pure RNA isolation kit (Roche, Hangzhou, China) according to the manufactory instructions. RNA was treated with DNase I and reverse-transcribed into cDNA using a Prime Script™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The resulting cDNAs were used as the template for expression detection analysis of the T6SS gene with qPCR using a SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) following the instruction of kit manual on an ABI
Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The gene-specific primer sequences are shown in Table 3. The average threshold cycle \((C_T)\) was used to determine the fold change of gene expression. In addition, 16S rRNA gene was used as an internal control. The \(2^{-\Delta\Delta C_T}\) method was used for relative quantification [49]. Each result represents the average of three independent determinations. This experiment was repeated twice.

4.6. Principle Component Analysis of Gene Expression

The PCA method was used for the data of T6SS gene expression under different conditions. The RE was subjected to PCA after standardizing to unit variance. Resulting factor scores of the PC1 and PC2 were tested in two-way analysis of variances (ANOVA). Data analyses were carried out using the SPSS 16.0 software (SPSS, Michigan Avenue, Chicago, IL, USA). The figures were produced by using the SigmaPlot 10.0 software (SYSTAT Software, Inc., San Jose, CA, USA).

4.7. Statistics Analysis

The software STATGRAPHICS Plus, version 4.0 (Copyright Manugistics Inc., Rockville, MD, USA) was used to perform the statistical analysis. Levels of significance \((p < 0.05)\) of main treatments and their interactions were calculated by analysis of variance after testing for normality and variance homogeneity.

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Author Contributions

Zhouqi Cui: designed methods and experiments, analyzed the data, and interpreted the results and wrote the manuscript. Guoqiang Jin: coordinated the project and provided financial support. Kaleem Ullah Kakar: carried out the laboratory experiments, analyzed array data and manuscript writing. Yangli Wang: coordinated the project and provided financial support. Guanlin Xie: coordinated the project and provided financial support. Guochang Sun: coordinated the project and provided financial support. Mohammad Reza Ojaghi an: helped with some laboratory works and writing the manuscript. Bin Li: conceived of the study, designed methods and experiments, wrote the manuscript, had final approval and takes responsibility for the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.
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