StoS, a Hybrid Histidine Kinase Sensor of *Xanthomonas oryzae* pv. *oryzae*, Is Activated by Sensing Low O₂ Concentration and Is Involved in Stress Tolerance and Virulence

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Bacteria have two-component signal transduction systems (TCSTS), which are important devices for receiving various environmental signals. A TCSTS generally consists of a sensor histidine kinase (HK) and a response regulator (RR) that contains a receiver domain. There are also hybrid-type HK (HyHK) that comprise a HK with a receiver domain within one molecule. In this study, we show that the deletion mutant of a HyHK *XOO_0635* (StoS) of *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight of rice, had decreased stress tolerance to high osmolarity, sodium, and H₂O₂. Growth of the StoS mutant was delayed, and viability was lower than the wild type in medium and in rice leaves. We found that StoS regulates the expression of various genes including *XOO_3715*, *XOO_0131*, and *stoS* itself. A domain search revealed a PAS domain with a heme pocket in StoS, implying that the HyHK functions as an O₂ sensor. When the bacteria were incubated in low oxygen, the StoS-dependent expression of *XOO_0131* and *XOO_3715* became higher. Therefore, StoS is activated by sensing a low O₂ concentration in its environs and is involved in gene expression for adapting to various stressful conditions.

Bacteria live in various environments, such as water, soil, plants, and animals, and are subjected to various stresses. Two-component signal transduction systems (TCSTS) are important devices to monitor and respond to environmental stimuli in bacteria (Laub and Goulian 2007; Parkinson and Kofoed 1992; Stock et al. 2000). A TCSTS generally consists of a membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). After detecting a stimulus, the HK can be autophosphorylated on a conserved histidine residue, and the phosphoryl group is transferred to an aspartic acid residue inside the N-terminal receiver domain of a cognate RR, resulting in the activation of the C-terminal output domain of the RR, which adaptively responds by modulating downstream genes and cellular machinery. In addition, Hybrid-type

HK (HyHK), which possess a sensor HK and receiver domains in one molecule, also exist.

TCSTS play important roles for controlling many bacterial physiological pathways, such as virulence, cell-to-cell signaling, nitrogen assimilation, osmolarity, and chemotaxis, in response to various environmental signals (Laub and Goulian 2007; Stock et al. 2000). Low oxygen concentration is a common stress that bacteria may encounter during growth or within a host. In *Rhizobium meliloti*, which live in symbiotic relationships with plants, low oxygen is a key signal to switch on nitrogen fixation (Ditta et al. 1987).

Oxygen sensing and transduction of the low oxygen signal have been well-studied in symbiotic bacteria. A pair of proteins named FixL and FixJ function as the HK and the corresponding RR, respectively (David et al. 1988; Virts et al. 1988). Protein FixL possesses a heme domain, a member of the PAS domain superfamily, that plays a key role for sensing oxygen. When oxygen binds to the heme domain on FixL, kinase activity is inhibited. On the other hand, dissociation of O₂ permits autophosphorylation at a histidine residue by using ATP, and the phosphoryl residue of the FixL is transferred to FixJ, which induces the expression of the genes involved in nitrogen assimilation under anaerobic conditions (Gilles-Gonzalez 2001).

An analysis of genome databases of various strains of xanthomonads, which comprise a major genus of phytopathogenic bacteria, predicted that they possess approximately 100 TCSTS-related genes in each genome (Bogdanove et al. 2011; Qian et al. 2008a), but only several of these have been assigned functions, such as RpfC/RpfG (Slater et al. 2000; Tang et al. 1991), RaxH/RaxR (Burdman et al. 2004), PhoP/PhoQ (Lee et al. 2008), ColS/ColR (Subramoni et al. 2012; Zhang et al. 2008), PdeK/PdeR (Yang et al. 2012), and HrpG (Wengelnik et al. 1996). Recently, He and associates (2009) showed that RavS, an HK protein harboring a heme pocket, and the cognate RR protein RavR play important roles for regulation of virulence-related gene expression through a global regulator Clp in *Xanthomonas campestris* pv. *campestris*, a bacterial pathogen causing black rot of crucifers. Qian and associates (2008b) conducted a genome-scale phenotypic characterization of RR in *X. campestris* pv. *campestris* and revealed that many TCSTS are involved in tolerance to various environmental stresses, such as high osmolarity, sodium stress, heat shock, and sodium dodecyl sulfate (SDS) exposure. However, which environmental signals are perceived by each sensor HK and the precise function of each RR, for the most part, remain unknown.

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Xanthomonas oryzae pv. *oryzae* is the causal agent of bacterial leaf blight of rice, one of the most important rice diseases wherever rice is grown (Niño-Liu et al. 2006; Ou 1985; Vauterin et al. 1995). According to the genome database of *X. oryzae* pv. *oryzae* MAFF311018 (Ochiai et al. 2005), the strain possesses 27 HK, 49 RR, and 14 HyHK (Bogdanove et al. 2011). A domain search predicted that, among these, six proteins (XOO_0308, XOO_0635, XOO_2200, XOO_2421 [a homolog to RavS], XOO_2639, and XOO_2988) harbor a heme domain, suggesting that the proteins function in response to O₂ concentration. In this study, we show that a novel HyHK, XOO_0635, is activated by low O₂ in the environment and is involved in bacterial virulence and tolerance to osmotic, sodium, and oxidative stresses. Here, we name the HyHK as StoS (stress tolerance-related oxygen sensor).

RESULTS

StoS is involved in tolerance to osmotic, sodium, and H₂O₂ stresses.

An open reading frame (ORF), XOO_0635, in *X. oryzae* pv. *oryzae* MAFF311018, which we named *stoS*, is predicted to encode an HyHK with both the sensor HK domain and the receiver domain. We generated X Δ StoS::Km, in which the almost entire region of *stoS* (+36 to +1,884 of 1,896 bp) is replaced with a kanamycin-resistance gene. To investigate phenotypic changes by the deletion of the ORF, we first examined tolerance of the mutant to high osmolarity, sodium stress, and H₂O₂. Bacterial strains that had been treated with 20% sorbitol or 0.5 M NaCl for 40 min were plated on peptone-sucrose (PS) medium, and subsequent colonies were counted (Fig. 1A).

Fig. 1. Stress tolerance of *Xanthomonas oryzae* pv. *oryzae* strains, the wild type with an empty vector (pHM1) (WT), the StoS mutant with pHM1 (Δ StoS) and the StoS mutant with pHMStoS (com) or pHMStoS Δ PAS (com-PAS). **A**, Bacterial populations after 40-min of exposure to high osmolarity (20% sorbitol) or sodium stress (0.5 M NaCl). Bacterial suspensions (10 μ l) including approximately 1×10^8 CFU were used for stress treatments, followed by dilution with 1 ml of distilled water (DW) in order to remove stresses. Bacterial strains were then plated on peptone-sucrose (PS) agar, and colonies were counted after 48 h. As a control, DW treatment was conducted. Means of three replications with standard deviations are shown. Different letters indicate significant differences ($P < 0.05$). Significance was determined by the Tukey's test. **B**, Growth inhibition zones of bacterial strains in which 3- μ l drops of various concentrations of H₂O₂ were dropped on PS agar plates. Bacterial strains were plated on PS agar, and 2.5, 5.0, 7.5, or 10.0 mM H₂O₂ was dropped onto the plate. Diameters of growth inhibition zones were measured after 3 days of incubation at 28°C. Means of three replications with standard deviations are shown. Values with a same letter do not differ significantly at the $P < 0.05$ level according to the Tukey's test.

Both treatments reduced viability of the mutant compared with that of the wild type, while little difference was observed between the wild type and the mutant when bacterial strains were treated with distilled water (DW) as a control.

To examine H₂O₂ tolerance, we plated bacterial strains on PS medium and dropped various concentrations (2.5 to 10 mM) of H₂O₂ on them. On the plate with the wild type, growth inhibition zones became larger, with increasing concentrations of H₂O₂ (Fig. 1B). Inhibition zones for the StoS mutant were larger than those of the wild type for each concentration, indicating lower H₂O₂ tolerance in the mutant than in the wild type.

The lower tolerance of the mutant strain to high osmotic stress, high salt concentration, and H₂O₂ was completely complemented by the introduction of the plasmid harboring *stoS* (pHMStoS) but not by the derivative (pHMStoSΔPAS), in which the part of the PAS domain containing the heme pocket is deleted (Fig. 1; Supplementary Figs. 1 and 2). The results suggest that StoS plays important roles in bacterial viability under various stresses, at least to osmotic, sodium, and H₂O₂ stresses, and that the PAS domain, especially the heme pocket in the domain, is indispensable for the StoS function.

Growth of StoS mutant in broth is delayed, probably because of low tolerance to H₂O₂ stress.

When bacterial strains were incubated in nutrient broth-yeast extract (NBY), growth of the StoS mutant was delayed, as compared with the wild type, and the delayed growth was complemented in XoΔStoS::Km (pHMStoS) (Fig. 2A). Bacteria produce H₂O₂ by their own metabolism, which might be an inhibitory factor for growth of the low H₂O₂ tolerance strain XoΔStoS::Km. To confirm the hypothesis, we compared the growth of the wild type and the mutant, using NBY broth with 0.1% sodium pyruvate, known to be a H₂O₂ remover (Mizunoe et al. 1999). As shown in Figure 2B, with the addition of sodium pyruvate, the growth curve of the mutant was very similar to that of the wild type, suggesting that StoS is involved in protecting the bacteria from the H₂O₂ that they produce by their own metabolism.

Lower viability of the StoS mutant in the late stationary phase.

The late stationary phase is expected to be a stressful condition for bacteria (Siegele and Kolter 1992), and we predicted that StoS may be involved in bacterial viability under such stress. To confirm this hypothesis, we grew bacteria in NBY broth (without sodium pyruvate) for 24 h, and then, dilution-plated a sample every 12 h until 96 h of incubation. Viability

of the wild type remained high until 84 h, after which it rapidly decreased (Fig. 3), whereas viability of XoΔStoS::Km had already decreased by 72 h. The viability of the complementary strain showed a pattern similar to the wild type. The results suggest that StoS is involved in bacterial viability in the stressful late stationary phase.

Reduced virulence of the StoS mutant.

Low viability and delayed growth in culture of the StoS mutant implied that the strain is less virulent. Susceptible rice cultivar IR24 was inoculated with a bacterial suspension, and lesion lengths were measured 14 days later. Although XoΔStoS::Km produced disease symptoms on rice leaves, lesions were shorter than those caused by the wild type and the complementary strain (Fig. 4A).

When bacterial numbers at the inoculation site (1 cm long leaf sections, including the inoculation site) were examined (Fig. 4B), the wild-type strain MAFF311018 (pHM1) and the complementary strain XoΔStoS::Km (pHMStoS) increased until 4 days after inoculation (dai), and they remained near their 4-dai level until 6 dai. In the case of the mutant strain XoΔStoS::Km (pHM1), bacterial numbers increased until 4 dai but were lower than those of the wild type and complementary strain, suggesting that bacterial growth of the mutant was inhibited in rice leaves, consistent with the lower virulence of the strain. Furthermore, the bacterial numbers in leaves inoculated with the mutant dropped rapidly after 4 dai compared with the wild type and the complementary strain. Similarly to culture, the mutant's low tolerance to stresses is likely to affect its growth and viability in infected rice leaves.

StoS is activated under the low O₂ condition.

Our preliminary microarray assay (*unpublished data*) revealed that the wild type and XoΔStoS::Km differed in the expression of various genes, including *XOO_0131* (predicted to encode the protein required for attachment to host cells; expression ratio of the wild type to mutant [wt/mt] = 16.86), *XOO_3715* (predicted osmotic shock protection protein with BON domain and homologous to a periplasm protein, OsmY, in *Escherichia coli* [Yim and Villarejo 1992]; wt/mt = 30.81), *XOO_0379* (catalase; wt/mt = 2.55), and *XOO_3220* (catalase; wt/mt = 6.55). Of these, we confirmed the StoS-dependent expression of *XOO_0131* and *XOO_3715*, using the β-glucuronidase (GUS) reporter assay and semiquantitative RT-PCR (semi-qRT-PCR). GUS activity of XoΔStoS::Km-derived transformants with a plasmid harboring the *gus* gene preceded by the putative promoter region of *XOO_0131* or *XOO_3715*

Fig. 2. Growth curves for *Xanthomonas oryzae* pv. *oryzae* strains, the wild type with an empty vector (pHM1) (WT), the StoS mutant with pHM1 (ΔStoS), and the StoS mutant with pHMStoS (com), in nutrient broth-yeast extract **A**, without or **B**, with 0.1% sodium pyruvate. Bacteria were incubated at 28°C, and the concentration (A₆₀₀) was measured every 12 h after inoculation. Three independent replications yielded similar results, and the representative data is shown.

(pHMXX00131::GUS or pHMXX03715::GUS, respectively) were specifically lower than that of the wild type with each corresponding plasmid (Table 1). Furthermore, results of the semi-qRT-PCR revealed less accumulation of *XOO_0131* and *XOO_3715* transcripts in the mutant than in the wild type, which were recovered by the introduction of the complementary plasmid pHMStoS (Fig. 5). The results suggest that StoS functions as a regulator of these genes and indicate that we could investigate the activation of the HyHK protein StoS by examining the expression of *XOO_0131* or *XOO_3715*.

Interestingly, GUS activity of the $Xo\Delta$ StoS::Km-derived transformant with pHMStoS::GUS, which harbors the *gus* gene preceded by the putative *stoS* promoter region, was also lower than that of the wild type-derived transformant (Table 1), suggesting that StoS regulates the expression of *stoS* itself.

In the sensor HK region, StoS possesses the PAS domain with a heme pocket, implying that the protein is probably activated by sensing low O_2 concentration as reported for FixL in *Rhizobium meliloti* and RavS in *X. campestris* pv. *campestris* (Gilles-Gonzalez 2001; He et al. 2009). The wild type-derived strain with pHMXX03715::GUS was incubated in NBY using flasks capped with aluminum foil (airtight) or a silicon cap (non-airtight) for 24 h, and the promoter activity of *XOO_3715* was assayed by measuring GUS activity (Fig. 6A). The activity was higher when the bacteria were incubated under the airtight condition than under the non-airtight condition.

For the semi-qRT-PCR analysis, moreover, the wild-type strain grown on NBY was prepared at an absorbance at 600 nm (A_{600}) = 1.0, and 1 ml of bacterial suspension was transferred to a 10-ml tube filled with N_2 (80%) + O_2 (20%), N_2 (100%), or CO_2 (100%), followed by incubation at 28°C for 15 min with shaking (120 rpm [BioShaker BR-23FP; Taitec, Koshigaya, Japan]). Then, total bacterial RNAs were extracted and used for semi-qRT-PCR analysis to examine the accumulation of *XOO_0131* and *XOO_3715* transcripts. As shown in Figure 6B, the expression level of *XOO_0131* and *XOO_3715* was higher when the bacteria were incubated under the no-oxygen conditions N_2 (100%) and CO_2 (100%). The higher ex-

pression of both genes under no-oxygen conditions was not observed for the StoS mutant. These results are confirmed by real-time quantitative RT-PCR analysis (Fig. 6C). Moreover, we found that the expression of *stoS* is also induced under the no-oxygen condition N_2 (100%) compared with the oxygen-containing condition N_2 (80%) + O_2 (20%), similarly to *XOO_0131* and *XOO_3715*. Although we could not examine the accumulation of *stoS* transcript in the *stoS* deletion mutant due to lack of the gene, the GUS reporter assay showed reduced expression of the *stoS*::*gus* fusion gene in the mutant, as described above (Table 1). Taken together, the results suggest that the HyHK protein StoS is activated through the sensing of a low O_2 concentration and, in turn, regulates various genes including *XOO_0131*, *XOO_3715*, and *stoS* itself.

DISCUSSION

To respond to and survive various environmental changes, bacteria must perceive a wide range of diverse environmental signals. TCSTS are important devices by which bacteria can sense such signals and then transduce them into either activating suitable gene expression, regulating physiological pathways for adapting to the environment, or both (Laub and Goulian 2007; Stock et al. 2000). The sensor domains in HK of TCSTS are categorized into 14 types (Ulrich et al. 2005). Of these, PAS domains are most frequently found and have been well-studied (Taylor and Zhulin 1999). PAS domains sense a wide range of environmental stimuli such as internal oxygen concentration, redox potential, and light (Taylor and Zhulin 1999). PAS domains contain cofactors (FAD, FMN, heme) bound to their central cavity, and in the case of HK that sense O_2 concentration, oxygen binds to the heme pocket. As reported for FixL in *Rhizobium* and *Bradyrhizobium* species and other O_2 sensors (Gilles-Gonzalez 2001), the release of O_2 from the heme pocket results in activation of the protein and the initiation of signal transduction.

In this study, we showed that in *X. oryzae* pv. *oryzae*, a hybrid type HK, StoS, with a heme pocket-containing PAS do-

Fig. 3. Viable numbers of *Xanthomonas oryzae* pv. *oryzae* strains, the wild type with an empty vector (pHM1) (WT), the StoS mutant with pHM1 (Δ StoS), and the StoS mutant with pHMStoS (com), in the late stationary phase. Bacterial strains were incubated in nutrient broth-yeast extract (without sodium pyruvate), with shaking, and were dilution-plated at 12-h intervals, starting at 24 h of incubation, to count viable bacteria. Mean viable bacterial numbers with standard deviations ($n = 3$ plates) per 1 μ l of culture were shown. Different letters indicate significant differences ($P < 0.05$), as determined by the Tukey's test. Three independent replications yielded similar results; representative data is shown.

main, functions as an O₂ sensor and that it is activated and allows the expression of downstream genes and of *stoS* itself in low O₂ conditions (Fig. 6). Protein StoS is involved in tolerance to various stresses, at least, osmotic, sodium, and oxidative stresses (Fig. 1). Growth of XoΔStoS::Km in culture was delayed compared with that of the wild type (Fig. 2A). Generally, bacteria produce H₂O₂ metabolically. And we showed that add-

Table 1. Mean β-glucuronidase (GUS) activity to evaluate expression of *XOO_0131::gus*, *XOO_3715::gus*, and *stoS::gus* in the wild type and StoS mutant (XoΔStoS::Km) strains of *Xanthomonas oryzae* pv. *oryzae*^a

Fusion gene	Wild type ^b	XoΔStoS::Km ^b
<i>XOO_0131::gus</i>	7.33 ± 1.18	0.06 ± 0.02
<i>XOO_3715::gus</i>	47.35 ± 2.49	0.29 ± 0.04
<i>stoS::gus</i>	0.99 ± 0.09	0.10 ± 0.04

^a pHMXOO0131::GUS pHMXOO3715::GUS or pHMStoS::GUS was used to transform the wild type and the StoS mutant, and transformants were incubated in nutrient broth-yeast extract broth for 16 h.

^b Shown as unit of GUS activity to microgram of bacterial inner proteins ± standard deviation; n = 3.

Fig. 5. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *XOO_0131* and *XOO_3715* expression in *Xanthomonas oryzae* pv. *oryzae* strains. WT = wild type with an empty vector (pHM1), ΔStoS = XoΔStoS::Km (pHM1), com = XoΔStoS::Km (pHMStoS). Total bacterial RNAs extracted after a 16-h incubation in nutrient broth-yeast extract were used for RT followed by PCR. 16S rRNA was used as a control. The PCR product obtained from the wild-type genomic DNA was used as a size marker.

Fig. 4. Virulence of *Xanthomonas oryzae* pv. *oryzae* strains, the wild type with an empty vector (pHM1) (WT), the StoS mutant with pHM1 (ΔStoS), and the StoS mutant with pHMStoS (com) on rice cultivar IR24. **A**, Lesion lengths were measured 14 days after inoculation. Values represent means with standard deviation from 20 leaves. Different letters indicate significant differences at the *P* < 0.01 level as determined by the Tukey's test. **B**, Viable bacterial numbers in rice leaves over time. Leaf sections (1 cm long, including the inoculated site) were homogenized and dilution-plated on nutrient broth-yeast extract medium with sodium pyruvate; colonies were counted after 2 days of incubation. Values represent means with standard deviation from four leaves. Different letters indicate significant differences (*P* < 0.05) on each day. Significance was determined using the Tukey's test.

ing sodium pyruvate, a known H_2O_2 remover, results in wild type-like growth of *XoΔStoS::Km* in the medium (Fig. 2B). It is likely, therefore, that StoS plays an important role for protecting *X. oryzae* pv. *oryzae* from H_2O_2 produced by the bacterium itself. Additionally, StoS is also likely to be involved in bacterial viability in the late stationary phase (Fig. 3), which is a highly stressful condition for bacteria (Siegele and Kolter 1992).

The StoS mutant was less virulent than the wild type on rice leaves (Fig. 4A). When we examined bacterial numbers in 1-cm-long leaf sections that included the inoculation site, we found that accumulation of the mutant was delayed during the early

infection stage (to 4 dai) and that bacterial viability rapidly decreased during late infection (from 5 dai) (Fig. 4B). Similar to growth in culture, the growth of the StoS mutant in the plant may be delayed because of its low tolerance to self-generated H_2O_2 . In addition, H_2O_2 and other reactive oxygen species accumulate in rice leaves infected with *X. oryzae* pv. *oryzae* as rice defense responses (Reimers et al. 1992; Sutherland 1991). So not only bacteria-generated H_2O_2 , but also reactive oxygen species derived from host rice plants may suppress growth of the low stress-tolerant strain *XoΔStoS::Km* during the early infection stage and its viability later in infection.

Fig. 6. Increased expression of *XOO_0131*, *XOO_3715*, and *stoS* in response to low O_2 conditions. **A**, The wild-type strain transformed with the β -glucuronidase (GUS) fusion plasmid was incubated in nutrient broth-yeast extract using flasks capped with aluminum foil (AT: airtight) or silicon cap (Non-AT: non-airtight) for 24 h, and GUS activity was measured. Numbers represent GUS activity (unit of GUS activity to microgram of bacterial inner proteins [U/μg]). **B**, Total RNA of the wild-type strain exposed to N_2 (80%) + O_2 (20%), N_2 (100%), or CO_2 (100%) was extracted and used for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Expression of each gene in the StoS mutant (Δ StoS) treated with 100% CO_2 was also tested. 16S rRNA was used as a control. PCR products amplified from the genomic DNA of MAFF311018 were used as size markers. +RT and -RT represent that reverse transcription was conducted or not prior to PCR, respectively. **C**, Real-time quantitative RT-PCR analysis. The wild type and the mutant strains were exposed to N_2 (80%) + O_2 (20%) or to N_2 (100%) for 15 min. Relative mRNA levels of the *XOO_0131*, *XOO_3715*, and *stoS* in each bacteria with respect to the wild type exposed to N_2 (80%) + O_2 (20%) are calculated. Values indicate means with standard deviation of triplicate measurements. Three independent replications yielded similar results, and the representative data is shown.

The StoS-deleted mutant maintains its virulence, although it is less than that of the wild type in our virulence test conditions. It is likely that the bacterium possesses functionally redundant genes. Alternatively, in nature, loss of StoS might result in serious damage to *X. oryzae* pv. *oryzae*, especially for bacteria that must multiply in an infected rice leaf and plant to serve as inoculum for the next infection cycle. Additionally, *X. oryzae* pv. *oryzae* must remain viable in unfavorable conditions, such as in dead leaves or diseased plants, soil, and water, so that the bacteria can enter new host plants and continue to spread in a field.

The genome database of *X. oryzae* pv. *oryzae* predicts that the bacterium possesses five other TCSTS-related genes that encode an O₂ sensor other than *stoS*. Of these genes, only *ravS* has been assigned this function (He et al. 2009). *RavS* in *X. campestris* pv. *campestris* is reported to be involved in regulating the expression of various virulence-related genes along with the counterpart RR protein *RavR*. The RR protein possesses an EAL domain, which functions in the catalysis of cyclic di-GMP to GMP. Cyclic di-GMP is known as a second messenger of bacteria and to inhibit the expression of the global regulator *Clp*, which is involved in expression of various virulence-related genes, such as extracellular polysaccharide and extracellular enzymes in *X. campestris* pv. *campestris* (Christen et al. 2005; Fineran et al. 2007; Schmidt et al. 2005; Tamayo et al. 2005). We tested for accumulation of *Clp* transcript in the StoS mutant but did not find any significant difference between the mutant and the wild type (data not shown).

X. oryzae pv. *oryzae* inhabits and grows in xylem vessels of rice, and the vessels become filled with bacteria and bacterial extracellular polysaccharides, preventing water flow. An increase in bacterial density and decrease in water flow must thereby lead to low O₂. Furthermore, bacterial growth also leads to plant defense responses including production of H₂O₂. To survive under such stresses and to be virulent on plants, the bacterium may activate stress tolerance genes via StoS by sensing low O₂ concentration as a signal.

HyHK possess both a HK domain and a receiver domain. A phosphorelay occurs between HyHK and the cognate RR protein. There are two types of HyHK; one has a histidine phosphotransfer (HPT) domain and the other does not (Zhang and Shi 2005). In the case of HyHK containing HPT, the phosphoryl residue is transferred from His in the HK domain to Asp in the receiver domain, and the phosphoryl residue is then transferred through the HPT domain to the receiver domain in the cognate cytoplasmic RR protein, which are found as paired HyHK/RR, e.g., ArcB/ArcA in *E. coli* (Ishige et al. 1994), BvgS/BvgA in *Bordetella pertussis* (Uhl and Miller 1996), and RpfC/RpfG in *X. campestris* pv. *campestris* (Slater et al. 2000). In the case of HyHK without the HPT domain, however, the phosphoryl residue at the Asp in the receiver domain is transferred to His of another protein containing the HPT domain and is subsequently transferred to the cytoplasmic RR protein, as found in Sln1-Ypd1-Ssk1 in *Saccharomyces cerevisiae* (Posas et al. 1996) and RcsC-YojN-RcsB in *E. coli* (Clarke et al. 2002; Takeda et al. 2001). StoS has no HPT domain, thus suggesting that at least two unknown proteins, an HPT-containing protein and an RR, may mediate the StoS-dependent expression of *XOO_3715* and *XOO_0131* and stress tolerance. Future studies are needed to reveal the entire cascade responsible for StoS-mediated regulation of the stress tolerance system in *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions.

Plasmids and bacterial strains used in this study are listed in Supplementary Table 1.

E. coli DH5 α MCR was cultured at 37°C in Luria-Bertani medium (Sambrook et al. 1989). *X. oryzae* pv. *oryzae* strains were usually grown at 28°C in NBY medium (Vidaver 1967) or in PS medium (Tsuchiya et al. 1985). All media were supplemented with the following antibiotics, as needed: ampicillin, 50 μ g/ml for *E. coli*; kanamycin, 25 μ g/ml for *X. oryzae* pv. *oryzae* and 50 μ g/ml for *E. coli*; spectinomycin, 25 μ g/ml for both *X. oryzae* pv. *oryzae* and *E. coli*.

Generation of *X. oryzae* pv. *oryzae* StoS mutant and the complementary strains.

A kanamycin-resistance gene derived from EZ::TN <KAN2> (Epicentre, Madison, WI, U.S.A.) flanked with a PCR-amplified 1,060-bp upstream region of *stoS* (*XOO_0635*), including the 35-bp 5' coding region and a 1,060-bp downstream region including the 13-bp 3' coding region, were cloned into pBluescript II KS+ (Stratagene, La Jolla, CA, U.S.A.). Supplementary Table 2 lists primers used in this study. Using the plasmid, marker-exchange mutagenesis was conducted on *X. oryzae* pv. *oryzae* MAFF311018 as described by Tsuge and associates (2001). The resultant mutant, in which the internal 1,847-bp fragment of the 1,896-bp *stoS* gene is replaced with the kanamycin-resistance gene, was confirmed by Southern blot analysis (data not shown) and was named Xo Δ StoS::Km.

A 2,904-bp *ApaI*-*SacI* fragment harboring *stoS* with the 423-bp upstream and the 585-bp downstream regions, prepared from an *stoS*-containing clone from the genomic library of MAFF311018, was cloned into the cosmid vector pHM1 (Innes et al. 1988). The resultant pHMStoS was introduced into Xo Δ StoS::Km, and the transformant was used for complementary tests.

Plasmid pHMStoS Δ PAS, which is similar to pHMStoS except for a deletion of +427 to +585 (+1 is A of a start codon of *stoS*.) corresponding to the conserved heme pocket domain, was also generated and was introduced into Xo Δ StoS::Km.

Assays for bacterial tolerance to osmotic, sodium, and H₂O₂ stresses.

Bacterial strains grown on NBY agar overnight were washed twice and were resuspended with DW to a concentration of A₆₀₀ = 0.3 (approximately 1 $\times$ 10⁸ CFU/ml). For the assay of bacterial tolerance to osmotic and sodium stresses, 1-ml samples of this suspension were centrifuged and bacterial pellets were resuspended in 10 μ l of 20% sorbitol, in 0.5 M NaCl, or in DW (as a control), followed by a 40-min incubation at room temperature. After the incubation, each sample was diluted with 1 ml of DW to stop stress treatments and was plated on PS agar to count surviving bacteria. For the assay of bacterial tolerance to H₂O₂ stress, 100- μ l samples of bacterial suspension (A₆₀₀ = 0.3) was plated on PS agar, and then, 3 μ l of 2.5, 5.0, 7.5, or 10.0 mM H₂O₂ solutions were dropped on the suspension. Plates were then incubated at 28°C for 3 days, growth inhibition zones that formed around the H₂O₂ spots were observed, and diameters of the inhibition zones were measured.

Assays for bacterial growth in the liquid medium.

Bacterial strains grown on NBY agar were washed twice and were adjusted to a concentration of A₆₀₀ = 1.0 with DW, 250 μ l of bacterial suspensions were added to 25 ml of NBY broth and, then, cultured at 28°C with shaking (120 rpm [Bio-Shaker BR-23FP]). Bacterial growth was examined by measuring the A₆₀₀ of culture every 12 h.

Bacterial viability tests in the late stationary phase.

Bacterial strains grown on NBY agar medium were washed twice and were adjusted to a concentration of A₆₀₀ = 1.0 with

DW, and 200 µl of the respective bacterial suspension was added to 20 ml of NBY liquid medium and cultured at 28°C with shaking (120 rpm [BioShaker BR-23FP]). At 12-h intervals after 24 h of incubation, 10 µl of culture was diluted and plated on NBY with 0.1% sodium pyruvate. After 2 days of further incubation, colony numbers were counted under the microscope.

Virulence test.

Bacterial strains grown on NBY agar medium were washed twice and were adjusted to a concentration of $A_{600} = 0.03$ with DW. Susceptible rice cultivar IR24 was inoculated with the bacterial suspension by leaf clipping (Kauffman et al. 1973), and lesion lengths were measured 14 days after inoculation. Bacterial numbers at the inoculation site were counted, using a 1-cm-long leaf section including the inoculated site. Each sample was homogenized with 1 ml of DW and was plated on NBY medium with sodium pyruvate. After incubation at 28°C, colonies grown were counted.

GUS reporter assay.

The 5' coding region and the preceding putative promoter region of *stoS* (−257 to +496), *XOO_0131* (−686 to +70), and *XOO_3715* (−912 to +13) were amplified by PCR. After treatment with appropriate restriction enzymes, each fragment was inserted in front of the promoterless GUS (*gus*) gene in pHMGUS (Tsuge et al. 2006) to produce pHMStoS::GUS, pHMXOO0131::GUS, and pHMXOO3715::GUS, respectively. We transformed XoΔStoS::Km and the wild type with each plasmid. GUS activity was measured as previously described (Jefferson et al. 1987; Tsuge et al. 2002). One unit of GUS activity was defined as nanomoles of *p*-nitrophenol released per hour. Also at this time, the concentration of bacterial inner proteins per assay was examined. Bacterial cells in 1 ml of culture were pelleted and resuspended with 100 µl of B-PER bacterial protein extraction reagent (Thermo Fisher Scientific, IL, U.S.A.) to extract bacterial proteins. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) and bovine serum albumin as a reference. GUS activity of each sample was calculated as units per microgram of bacterial inner proteins.

Semi-qRT-PCR and real-time qRT-PCR.

Bacterial total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany). After the quality of the RNA sample was confirmed by agarose gel electrophoresis, 0.2 µg of each RNA sample was used for synthesis of cDNA, using a reverse-transcriptase ReverTra-Ace (Toyobo, Osaka, Japan). For semi-qRT-PCR, a DNA polymerase blend *Taq* (Toyobo) was used, and amplified fragments were separated by agarose gel electrophoresis, followed by visualization with ethidium bromide. Real-time qRT-PCR was performed on the Roter-Gene Q system (Qiagen) using Thunderbird SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions. The comparative quantitation method was used to calculate the relative mRNA level with respect to the corresponding transcript in bacteria cultured in the normal air condition N₂ (80%) + O₂ (20%). As a control, 16S rRNA was used.

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