Enhanced Control of Plant Wilt Disease by a Xylose-Inducible *degQ* Gene Engineered into *Bacillus velezensis* Strain SQR9XYQ

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ABSTRACT

Bacillus velezensis SQR9 (former *B. amyloliquefaciens* SQR9) is a plant-growth-promoting rhizobacterium (PGPR) that promotes plant growth and health. The colonization of PGPR strains along plant roots is a prerequisite for them to execute their specific functions. However, one problem of microbial introduction in practice is that the applied PGPR strains do not always successfully colonize the rhizosphere. In *Bacillus* spp., two-component signal transduction system (TCS) DegS/U regulates flagellar motility, biofilm formation and antibiotic production. Phosphorylation of DegU by DegS is positively affected by DegQ protein. In this

study, we constructed a xylose-inducible *degQ* genetically engineered strain SQR9XYQ to improve the biocontrol activity. The results from in vitro, root in situ, greenhouse experiments and RT-qPCR studies demonstrate that (i) the phosphorylation of DegU in SQR9XYQ can be gradually activated by xylose, which is a component of both cucumber and tomato root exudates, and (ii) biofilm formation, antibiotic expression, colonization activity, and biocontrol efficiency were improved in SQR9XYQ compared with the wild-type strain SQR9. These results suggest that colonization trait is important to biocontrol strains for maintenance of plant health.

Plant vascular wilt diseases caused by soilborne pathogenic microorganism are economically important diseases throughout the world. Some examples are fungal wilt pathogen *Fusarium oxysporum* f. sp. *cucumerinum* J. H. Owen and bacterial wilt pathogen *Ralstonia solanacearum* cause wilt diseases of cucumber and tomato, respectively (Freeman et al. 2002; Swanson et al. 2005). Biological control represents a promising approach for protection of plants against soilborne pathogens (Ongena and Jacques 2008). The gram-positive *Bacillus velezensis* is a rhizobacterium that promotes plant growth and health (Ongena and Jacques 2008). In addition, *B. velezensis* SQR9 and FZB42 are used commercially as biofertilizers and biocontrol agents in agriculture (Cao et al. 2011; Chowdhury et al. 2015). However, the signaling networks involved in the beneficial microbe–plant interactions in the rhizosphere remain largely unknown, and this hampers their use in the field. Among the difficulties encountered, the maintenance of a sufficient microbial population at the root surface or within the roots is a critical issue.

Root exudates represent an important source of nutrients for microorganisms in the rhizosphere and seem to participate in early colonization-inducing chemotactic responses of rhizosphere bacteria (Bais et al. 2006; Bacilio et al. 2003). Surprisingly, so far, only a few plant signaling molecules affecting rhizosphere microbiology have been studied and elucidated in detail. For

example, the most common quorum sensing (QS) system in gram-negative bacteria uses N-acyl homoserine lactone (AHL) as a signal (Fuqua et al. 2001). In *Bacillus*, the transcriptome profile was generated in experiments where root exudate stimulations were used to mimic interactions between bacteria and plants, it was found that the transcriptional factor DegU plays an important role in gene regulations during plant colonization (Fan et al. 2015; Mwita et al. 2016). Engineering the interactions between the plants and microorganisms may also be a promising approach to develop biocontrol procedures. In general, the carbohydrates found in root exudates include arabinose, glucose, galactose, fructose, sucrose, pentose, rhamnose, raffinose and xylose (Bacilio et al. 2003). A xylose-inducible promoter system is a promising candidate to improve the interactions between plants and microorganisms (Kim et al. 1996; Radha and Gunasekaran 2007).

Two-component signal transduction systems (TCS) allow bacteria to sense and respond to the environment (Murray et al. 2009). The genome of *B. velezensis* SQR9 encodes 30 TCS. These signal transduction pathways are involved in regulating a number of diverse cellular processes that allow *B. velezensis* to efficiently adapt to its surroundings (Cairns et al. 2015). One such TCS is DegS–DegU, involving the histidine sensor kinase DegS and the response regulator DegU, which plays an important role in regulating multicellular behavior and antibiotic biosynthesis (Xu et al. 2014). In addition, DegS–DegU is modulated by DegQ, a small polypeptide of 46 amino acids that enhances the phosphorylation of DegU (DegU:P) (Verhamme et al. 2007). Previous research demonstrated that the regulatory control exerted by DegU is more flexible than a simple molecular switch and has the capability to integrate physiological responses along a gradient of DegU:P (Kobayashi 2007). In *Bacillus*, swarming motility is activated by very low levels of DegU:P, and complex colony architecture is activated when they increase the levels of DegU:P (Kobayashi 2007). In addition, the gradual phosphorylation of DegU in *B. velezensis* SQR9 can influence biocontrol activity by increasing rhizosphere colonization and regulating the synthesis of

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bacillomycin D (Xu et al. 2014). These data suggest that the maintenance of an appropriate level of DegU:P in the cell facilitates the colonization process of the plant root in the soil. Moreover, DegU was found to be involved in nonribosomal synthesis of antimicrobial transcription secondary metabolites in endospore-forming Gram-positive soil bacteria. Several studies have elucidated that DegU regulates bacillomycin D and bacilysin by binding its promoter in *B. velezensis* FZB42 (Koumoutsis et al. 2007; Mariappan et al. 2012). It has also been reported that a *degU*-negative mutant strain which was found to be impaired in bacilysin and difficidin synthesis (Mariappan et al. 2012), indicating that the global regulator DegU was required for production of these antibiotics.

Although *Bacillus velezensis* SQR9, isolated from the rhizosphere of cucumber, can directly suppress the growth of *Fusarium oxysporum* and stimulate plant growth, when this strain was applied in the field, the populations of strain SQR9 in the rhizosphere was unstable (Cao et al. 2011). In this study, we used a recombinant *degQ* gene under the control of a xylose inducible promoter (*Pxyl*) in *B. velezensis* SQR9, which can use xylose in the root exudates as a signal in the rhizosphere. Our results suggest that this engineered strain improved the root colonization ability and biocontrol activity by altering the phosphorylation level of DegU in the cell.

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* Top 10 (for the preparation of plasmids) and *B. velezensis* strain SQR9 (CGMCC accession no. 5808, China General Microbiology Culture Collection Center; NCBI accession CP006890) cells were grown at 37°C in Luria-Bertani (LB) medium. *F. oxysporum* CIPP1012 (ACCC accession no. 30220, Agricultural Culture Collection of China) was used as the pathogen and was obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (Beijing, China). The pathogenic *R. solanacearum* strain QLRs-1115 was previously isolated from a wilted tomato plant (Xu et al. 2014). For biofilm formation, *B. velezensis* and its derived strains were cultivated in MSgg medium (5 mM potassium phosphate, 100 mM MOPS, pH 7, 2 mM MgCl₂, 700 μM CaCl₂, 50 μM MnCl₂, 50 μM FeCl₃, 1 μM ZnCl₂, 2 mM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg ml⁻¹ tryptophan, 50 μg ml⁻¹ of phenylalanine, and 50 μg ml⁻¹ of

threonine). Minimal medium-glucose-yeast (MGY) medium contained (g/liter) glucose (5), yeast extract (4), NH₄NO₃ (1), NaCl (0.5), K₂HPO₄ (1.5), KH₂PO₄ (0.5), and MgSO₄ (0.2). MGY-Cl medium was an MGY-based medium supplemented with 5 mM *p*-Cl-Phe (Zhou et al. 2016). When required, antibiotics were added to media at the following final concentrations: ampicillin at 100 μg ml⁻¹, chloramphenicol (Cm) at 5 μg ml⁻¹, and kanamycin at 30 μg ml⁻¹.

Deletion of the *degQ* gene in *B. velezensis* SQR9. The marker-free deletion strain of the *degQ* gene were constructed using the *Pbc-pheS**-cat (PC) cassette and overlap-PCR based strategy essentially as previously described (Zhou et al. 2016). The construction of *Pbc-pheS**-cat (PC) cassette was carried out as described by Zhou et al. (2016). The left flanking (LF) region (:900 bp), direct repeat (DR) sequence (:800 bp), and right flanking (RF) region (:500 bp) were amplified from strain SQR9 using the primer pairs p3/p4, p5/p6, and p7/p8, respectively. The PC cassette was amplified with the primer pair p9/p10 using pTPC as a template. These four fragments were fused using overlap PCR in the order of the LF, DR, PC cassette, and RF. The resulting *degQ* deletion amplicon (PCR production) was directly transformed into strain SQR9, and the transformants were selected on LB plates containing Cm. The chloramphenicol resistance (Cm^r) colonies were cultivated to an OD₆₀₀ of 1.0 without Cm, and a 100-μl aliquot of a 10-fold dilution of cultures (approximately 10⁵ cells) was plated on MGY-Cl medium. Mutants growing on MGY-Cl were further confirmed by DNA sequencing and named Δ*degQ*.

Construction of the xylose-inducible *degQ* genetically engineered *B. velezensis* strain SQR9XYQ. The construction of the xylose-inducible *degQ* genetically engineered strain was performed in the *degQ* marker free deletion mutant Δ*degQ*. Briefly, the *amyELF*, *amyERF*, and *degQ* genes were amplified from strain SQR9 using the primer pairs p11/p12, p13/p14, and p15/p16, respectively. Cm^r gene was amplified from plasmid Pnw33 N. Finally, the xylose-inducible *Pxyl* promoter was amplified from the plasmid PWH1510 using primer pairs p17/p18. The five fragments were fused using overlap PCR in the order of *amyELF*, *Pxyl*, *degQ*, Cm^r, and *amyERF*, which was directly transformed to Δ*degQ*. Transformants were selected on LB plates containing Cm, further confirmed by DNA sequencing and named SQR9XYQ.

Biofilm assay of *B. velezensis* SQR9 and its derivative strains. The biofilm assay was carried out using a modified version of the microtiter plate assay as described by Hamon and Lazazzera (2001). Briefly, *B. velezensis* cells were grown overnight at 30°C on

TABLE 1. Microorganisms and plasmids used in this study

Strain	Genotype	Reference
<i>F. oxysporum</i> f. sp. <i>cucumerinum</i> J. H. Owen (FOC NJAU-2)		ACCC
<i>E. coli</i> top 10	F-mcrAΔ (mrr-hsdRMS-mcrBC) ψ80lacZAM15Δ lacX74 nupG recA1 araD139Δ (ara-leu) 7697 galE15 galK 16 rpsL (StrR) end A1λ-	Invitrogen (Shanghai)
<i>R. solanacearum</i> QLRs-1115		Laboratory stock (Xu et al. 2014)
<i>B. velezensis</i> SQR9	Wild type	Laboratory stock (Xu et al. 2014)
<i>B. velezensis</i> SQR9- <i>gfp</i>	GFP labeled <i>B. velezensis</i> SQR9, Kan ^r	Laboratory stock (Xu et al. 2014)
Δ <i>degQ</i>	<i>B. velezensis</i> SQR9 Δ <i>degQ</i>	This study
Δ <i>degQ</i> - <i>gfp</i>	GFP labeled <i>B. velezensis</i> SQR9 Δ <i>degQ</i> , Kan ^r	This study
SQR9XYQ	<i>B. velezensis</i> SQR9Δ <i>degQ</i> , <i>amy</i> :: <i>Pxyl</i> - <i>degQ</i> ::Cm	This study
SQR9XYQ- <i>gfp</i>	GFP labeled <i>B. velezensis</i> SQR9 Δ <i>degQ</i> , Kan ^r Cm ^r	This study
ATCC14579	<i>B. cereus</i> , wild type	ATCC
Plasmid		
pMD 19-T	Ap ^r ; TA clone vector	Invitrogen (Shanghai)
pNW33n	Cm ^r ; <i>E. coli</i> - <i>Bacillus</i> shuttle vector	BGSC
pHapII	pUBC19 containing <i>gfp</i> fused to HAPII promoter	Laboratory stock
pWH1510	Ap ^r ; <i>xylA</i> -lacZ	(Rygus et al. 1991)
pTDC	pMD19-T harboring the PC cassette	(Zhou et al. 2016)
pNZT1	Em ^r ; temperature-sensitive <i>E. coli</i> - <i>Bacillus</i> shuttle vector	(Zhou et al. 2016)
pNZT1- <i>pheS</i> *	pNZT1 with <i>Pbc-pheS</i> * cassette inserted in the multicloning sites	(Zhou et al. 2016)

LB plates until they were used to inoculate in MSgg liquid medium, which was then incubated with shaking. Next, the strains were cultured to the same density ($OD_{600} = 1.0$), and the cells were collected by centrifugation, washed twice with MSgg medium and resuspended in the same volume of MSgg medium. One hundred microliter of the suspension was separately inoculated into 24-well polyvinylchloride (PVC) microtiter plates (Fisher Scientific) filled with 2 ml of MSgg medium. The microtiter plates were incubated at 30°C without shaking. After incubating strains in 24-well microtiter plates for various periods, the presence of adherent biofilm cells was evaluated by crystal violet (CV) staining. Growth media and nonadherent cells were removed from the microtiter plate wells, which were subsequently rinsed with washing buffer (0.15 M ammonium sulfate, 100 mM potassium phosphate, pH 7, 34 mM sodium citrate, and 1 mM $MgSO_4$). Biofilm cells were stained with 1% CV in washing buffer at room temperature for 20 min. Excess CV was subsequently removed, and the wells were rinsed with water. The bound CV was solubilized with 200 μ l of 80% ethanol-20% acetone. The number of biofilm cells were quantified by using a multifunctional plate reader Spectra Max M5 analysis system (Molecular Devices Corporation, CA) to measure the OD_{570} value of each well.

Gene transcription analysis by RT-qPCR. Total RNA was extracted from *B. velezensis* strains (SQR9, $\Delta degQ$, and SQR9XYQ) grown in MSgg and MS medium by using the E.Z.N.A. bacterial RNA kit (OMEGA, Biotek). Reverse-transcription of RNA was conducted with PrimeScript RT reagent kit with a gDNA eraser (TaKaRa, Biotek, China). Transcription levels of *yvcA* and *aprE* of $\Delta degQ$ and SQR9XYQ relative to SQR9 were measured by reverse transcription quantitative PCR (RT-qPCR) using a SYBR Premix Ex TaqTM (Perfect Real Time) Kit (TaKaRa, Dalian, China). The sequences of primers used to amplify these genes are listed in Supplementary Table S1. The *recA* gene was used as the internal control. Reactions were carried out on an ABI 7500 system (ABI) under the following conditions: cDNA was denatured for 10 s at 95°C, followed by 40 cycles, consisting of 5 s at 95°C and 34 s at 60°C. The $2^{-\Delta\Delta Ct}$ method was used to analyze the RT-qPCR data (Livak and Schmittgen 2001).

Microscopy. Laser scanning confocal microscope (LSCM) images of plant roots colonized by tested strains were captured with a Leica confocal microscope (Leica Model TCS SP2, Heidelberg, Germany). In the hydroponics system, the root samples were monitored at 2 and 4 days after inoculation. Excitation of the samples was performed at 488 nm for chimeric proteins fused to GFP. Fluorescent GFP emission was collected using a combination of filters (SP 555) and a beam splitter (549 nm). Images were assisted with Leica confocal software, version 2.61.

Determination of xylose content in plant root exudates. The concentration of xylose in different plants (cucumber, tomato, and banana) root exudates was measured using the MBTH method (Anthon and Barrett 2002). Cucumber seeds (Jinchun NO.4), tomato seeds (*Lycopersicon esculentum* 'Jiangshu') and banana seedlings (Musa AAA Cavendish cultivar Brazil) were used for the root exudate collection. After 1 month of culturing in the sterile tissue-culture container, the plants were placed into 50-ml flasks with sterilized double-distilled water and cultured at 28°C with a 16-h light and 8-h dark photoperiod for 4 days. Root exudates were collected every day during the 4-day period. The combined solutions (1,000 ml from the 20 individual plants) were filtered through a 0.45 μ m membrane filter (Millipore, Billerica, MA), and the sterility of the root exudates was determined by plating 100 μ l exudates on LB medium and incubating them at 30°C for 24 h. The filter-sterilized root exudates were lyophilized. The dry weights of the roots in each sample were computed to standardize and compare the biological data from the test. A 40- μ l aliquot of the redissolved root exudates was mixed with 60 μ l of H_2O , 100 μ l of 0.5 M NaOH, and 100 μ l of MBTH buffer containing 3-methyl-2-benzothiazolinon hydrazine (MBTH) at 1.5 mg/ml and DTT at

0.5 mg/ml. The samples were heated to 80°C for 15 min, followed by the addition of 200 μ l of a solution containing 0.5% sulfamic acid, 0.5% $FeNH_4(SO_4)_2$, and 0.25 M HCl. The xylose concentrations were then determined at 620 nm.

Root colonization by *B. velezensis* SQR9 and its derivative strains in hydroponic culture. The shuttle plasmid pHAPII was used to introduce plasmid-borne GFP genes into *B. velezensis* SQR9 and its derivative strains by electroporation (Cao et al. 2011). To study root colonization, *B. velezensis* strains were labeled with GFP to enable their monitoring. Cucumber seeds were surface-sterilized, germinated in axenic conditions and grown in sterile vermiculite. When two cotyledons emerged, the cucumber seedlings were washed carefully in sterile water to remove the vermiculite and cultured in 50 ml of 1/4 sterile MS medium until the seedlings had three to four true leaves. Colonies of *B. velezensis* strains (SQR9-*gfp*, $\Delta degQ$ -*gfp*, and SQR9XYQ-*gfp*) were inoculated into 50 ml of LB broth with appropriate antibiotics, and the cultures were incubated at 30°C until they reached the stationary phase. The cells were collected and washed twice in M8 buffer (22 mM Na_2HPO_4 , 22 mM KH_2PO_4 , and 100 mM NaCl, pH 7) and resuspended in 500 ml of M8 buffer prior to use. Axenically prepared cucumber seedlings were soaked in 500 ml of bacterial suspensions for 30 min at 30°C. Subsequently, seedlings were aseptically transplanted to containers with 200 ml of sterile 1/2 MS culture medium. The plants were incubated in a growth chamber at 28°C with a 16-h light regimen. Ten replicates were performed for each strain.

To monitor the *B. velezensis* strains colonized on cucumber seedling roots, root samples were taken at 2 and 4 days after inoculation, and 0.2 g of roots was homogenized in 1.8 ml of PBS buffer using a mortar and pestle until a fine homogenate was obtained. The homogenates were serially diluted and plated onto LB plates with the appropriate antibiotics. After culture at 30°C for 2 and 4 days, the bacterial colonies were examined using fluorescence microscopy (Olympus DP71), and those emitting green fluorescence were counted.

Greenhouse experimental design. The trial was conducted from 11 September to 19 November 2017 in the greenhouse at Nanjing Agricultural University. The soils used for the pot experiments were collected from a loamy field. The field is located in Nanjing, Jiangsu Province, China, and the soil had the following properties: pH 6.1, organic matter 8.3 g kg^{-1} , available N 73.6 mg kg^{-1} , available P 27.2 mg kg^{-1} , and available K 38.7 mg kg^{-1} .

Seeds of cucumber (Jinchun NO.4) and tomato (*Lycopersicon esculentum* 'Jiangshu') were surface-disinfected in 2% sodium hypochlorite for 3 min, rinsed three times in sterile distilled water and subsequently allowed to germinate in 9-cm Petri dishes covered with sterile wet filter paper at 28°C. After germinating, the seeds were planted in seedling trays. After 25 days, the seedlings were transplanted into larger pots with 10 kg of soil. Four treatments for each plant in the pot experiment were designed as follows: CK (control), SQR9, $\Delta degQ$, and SQR9XYQ. All strains were individually mixed into the soil as follows: *F. oxysporum* f. sp. *cucumerinum* at 10^5 spores g^{-1} of soil for cucumber, *R. solanacearum* strain QLRs-1115 at 10^6 cfu g^{-1} for tomato, and *B. velezensis* strains (SQR9, $\Delta degQ$, and SQR9XYQ) at 10^8 cfu g^{-1} . The treatment without *B. velezensis* strains was used as the control. Each treatment was replicated 30 times, which included three blocks in a completely randomized design (10 plants for each block). The seedlings were incubated in a greenhouse with a temperature of 21 to 33°C and a relative humidity of 62 to 85%.

The disease index was recorded on each individual plant from 6 days to 9 days after the *F. oxysporum* f. sp. *cucumerinum* inoculation and expressed on a scale of 0 to 4 as follows: 0, the entire plant was healthy; 1, <25% of leaves were wilted; 2, 25 to 50% of leaves were wilted; 3, 50 to 75% of leaves were wilted; and 4, 75 to 100% of leaves were wilted. The disease index for each treatment was calculated using the following formula (Peltier and Grau 2008):

Disease index = $[\sum(\text{rating} \times \text{number of plants rated}) / (\text{total number of plants} \times \text{highest rating})] \times 100$.

Statistical analysis. The data were statistically analyzed using analysis of variance, followed by Fisher's least significant difference test ($P < 0.05$) using SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Biofilm formation activity of the *degQ* gene engineering strain SQR9XYQ can be gradually activated by xylose. Based on the specific function of the small protein DegQ on DegU: P, we constructed a recombinant *degQ* gene under the control of a xylose-inducible promoter in two steps. First, a marker-free deletion of the *degQ* gene was constructed by a *pheS** and overlap-PCR-based strategy to avoid the complication of having two copies of *degQ* genes under the control of different promoters (Fig. 1A). Second, the *degQ* gene was fused to the *xyl* promoter then inserted into a 4.0-kb fragment and recombined at the *amyE* locus (Fig. 1B). To investigate whether the *degQ* gene is tightly regulated by the *P_{xyl}* promoter, the expression of the genes *yvcA* and *aprE*, which are known to be activated by DegU: P, were monitored by RT-qPCR at increasing concentrations of xylose (Fig. 1C). The results showed that the highest transcription levels of *yvcA* and *aprE* were detected after the addition of 0.9% xylose, followed by the addition of 0.6% xylose and 0.3% xylose, respectively. As a negative control, the transcripts of *yvcA* and *aprE* in a $\Delta degQ$ mutant decreased significantly, with average transcription levels that were 15- and 1.7-fold lower, respectively (Fig. 1C). These data suggest that the phosphorylation of DegU in SQR9XYQ can be gradually activated by xylose.

DegU: P is required to regulate complex colony architecture and biofilm formation in the wild-type SQR9 strain (Xu et al. 2014). In Supplementary Figure S1, the wild-type SQR9 produced colonies with complex architecture features on solid media, and the morphology of the SQR9 colonies was not affected by increasing the concentrations of xylose from 0 to 0.9%. As expected, the colonies of strain SQR9XYQ showed a similar pattern to that of the $\Delta degQ$ mutant strain in the absence of xylose in the medium (Fig. 2A). Both SQR9XYQ and the $\Delta degQ$ mutant strain showed impairment in the structure of colony architecture. In addition, we observed extended formation of the aerial colony architecture of the SQR9XYQ strain compared with the results of the wild-type strain on agar plates containing 0.6% or 0.9% xylose. These results demonstrated that the colony complex architecture features of strain SQR9XYQ were increased by increasing the concentration of xylose in the culture media.

A comparable induction pattern by the SQR9XYQ strain was observed in the biofilm formation process at increasing concentrations of xylose, although no effect of the xylose concentration on growth was observed in the growth curves. This pattern correlated well with the measured values of pellicle biomass (Fig. 2B), thus reinforcing the observation that the biofilm formation activity of SQR9XYQ strain can be activated by xylose in the medium.

Comparison of xylose content in the root exudates of cucumber, tomato and banana plants. In this study, xylose was used as a signal to promote the colonization of strain SQR9XYQ on the root surface. Therefore, we investigated the concentration of xylose in the root exudates of cucumber, tomato, and banana plants. The quantification results showed that cucumber plants secreted the highest levels of xylose, followed by tomato and banana plants after 4 days of collection. Specifically, the xylose concentration in the cucumber root exudates was 21.56 $\mu\text{g/ml}$, which was 8- and 10-fold higher than those in tomato and banana, respectively (Fig. 3A). These results suggest that the cucumber rhizosphere can provide a better microhabitat for strain SQR9XYQ to exert its biocontrol activity. All three types of plant root exudates can stimulate the biofilm formation of strain SQR9XYQ, and

cucumber root exudates promoted biofilm formation the most strongly (Fig. 3B).

In situ observation of the xylose-induced rhizosphere colonization on cucumber root. To further investigate whether xylose in the hydroponics system affects the root colonization process of SQR9 and its derivative strains, their degree of colonization on the roots of cucumber seedlings were monitored. After 4 days post-co-cultivation of cucumber roots and green fluorescent protein (GFP)-labeled *B. velezensis* strains (SQR9-*gfp*, SQR9XYQ-*gfp*, and $\Delta degQ$ -*gfp*) in a hydroponic system, both live

Fig. 1. Graphic representation of the construction of the genetically engineered strain SQR9XYQ. **A**, To delete the target gene *degQ*, an approximately 800-bp fragment just downstream of the target region is used as a direct repeat (DR) sequence and is placed ahead of the PC cassette. Then, transformants with an insertion of the fragment containing the DR and PC cassette just upstream of the target region are selected on Cm. Finally, recombination between two DR sequences excises both the PC cassette and the target fragment, and the resulting mutant is selected on 5 mM *p*-Cl-Phe. **B**, The construction of the recombination *degQ* gene under the control of *P_{xyl}* at the *amyE* locus of the *Bacillus velezensis* chromosome by using overlap-PCR. **C**, Transcriptional levels of *yvcA* and *aprE* during increasing xylose concentrations in different *B. velezensis* strains evaluated by RT-qPCR.

and dead roots (cucumber seedlings without stem and leaves) were viewed using confocal scanning laser microscopy (CSLM). In the dead root system, we compared the colonization ability of SQR9-*gfp* and SQR9XYQ-*gfp* strains when subjected to different concentrations of xylose ranging from 0 to 0.9% in the medium. The result showed that the colonization pattern of the wild-type SQR9-*gfp* strain was not affected by xylose concentration (Fig. 4A to D). However, increasing the xylose concentration in the medium significantly improved the root colonization activity of the SQR9XYQ-*gfp* strain, indicating that xylose can be an important signal for SQR9XYQ (Fig. 4E to H). SQR9XYQ strains in the typical hydroponic system heavily colonized a segment of the primary root located approximately 2 to 4 cm from the root tip. However, only a few $\Delta degQ$ -*gfp* strains colonized on root was observed compared with other strains in the same position (Fig. 4I to L).

The rhizosphere colonization activity and antibiotic gene expression were improved by recombinant *degQ* gene under the control of *xyl* promoter. The colonization of plant-growth-promoting rhizobacterium (PGPR) strains can change rapidly over time in the rhizosphere, so we performed a quantitative analysis on the population of wild-type and SQR9XYQ strains on plant roots at 2 and 4 days after inoculation in the hydroponic system. The result showed that the population of the SQR9XYQ strain significantly increased as compared with the wild-type strain on both cucumber and tomato roots by using colony counting method, which suggests that strain SQR9XYQ is the better colonizer (Fig. 5A and B). In addition, the transcription of the antibiotic encoding genes was monitored by RT-qPCR from cells colonized on the plant root. Compared with SQR9, the *bamD* and *dfnA* transcriptions in strain SQR9XYQ increased sixfold and fourfold from cucumber roots, respectively (Fig. 5C). A similar result was observed in the *B. velezensis* cells collected from tomato roots. Moreover, in a competition colonization assays, recombinant *degQ* gene under the control of a xylose inducible promoter in *amyE* locus improve the competitive ability of

B. velezensis SQR9 in the cucumber rhizosphere at 2 and 4 days (Supplementary Fig. S2).

Application of strain SQR9XYQ can improve the biocontrol efficacy of both cucumber and tomato wilt disease. The results presented above showed that the *degQ* genetically engineered strain SQR9XYQ can efficiently colonize plant roots by sensing xylose in the root exudates. In addition, the expression of the antibiotic bacillomycin D was improved since DegU directly binds to and activates the expression of *Pbmy*, which is the promoter of the bacillomycin D operon. We predicted that the strain SQR9XYQ with a high level of DegU:P in the rhizosphere would perform better as a biocontrol agent against pathogens. As expected, there were significant differences between the treatment of the control, SQR9, $\Delta degQ$ and SQR9XYQ in cucumber greenhouse experiments. The disease incidence of $\Delta degQ$ treatment averaged 70.7%. Treatment with SQR9 showed an average disease incidence of 43.2%. Treatment with the SQR9XYQ strain demonstrated a disease incidence of 20.4%, which was significantly lower than that of the wild-type strain. Application of the SQR9XYQ strain can slightly improve the biocontrol activity with an average disease incidence of 36.7% compared with an average disease incidence of 44.5% when SQR9 was applied in the tomato greenhouse experiment (Fig. 6).

DISCUSSION

The colonization activity of PGPR strains along plant roots is a prerequisite to suppressing infection by pathogens and reducing the incidence of disease (Compant et al. 2005). Our previous research

Fig. 2. Increasing levels of xylose concentration in the medium improved the complex colony morphology and pellicle formation activity of *Bacillus velezensis* SQR9XYQ. **A**, Upper panel: representative images showing the complex architecture of strain SQR9XYQ under increasing xylose concentrations on solid MSgg medium after 24 h incubation. Lower panel: pellicle formation of strain SQR9XYQ under increasing xylose concentrations in liquid MSgg medium after 24 h incubation. **B**, OD₅₇₀ of solubilized crystal violet from microtiter plate assay over time for *B. velezensis* SQR9XYQ strains with the concentrations of xylose indicated.

Fig. 3. **A**, Xylose detection in cucumber, tomato, and banana root exudates. The xylose concentration in the root exudates was measured using the MBTH method. The root exudates were collected in the sterile tissue-culture container, and the dry weights of the roots in each sample were computed to standardize and compare the biological data from the test. **B**, Representative images showing the pellicle formation activity of strain SQR9 and SQR9XYQ in microtiters by adding different root exudates into MSNc liquid medium.

showed that increased levels of DegU phosphorylation in *B. velezensis* cells could improve its root colonization efficiency and biocontrol activity (Xu et al. 2014). In this study, our goal was to construct a potential way to recruit designated plant beneficial rhizobacteria for plants. Thus, a xylose-inducible promoter (*P_{xyI}*) was used to induce the expression of the *degQ* gene to facilitate the utilization of this resource in the root exudates. The results showed that the biofilm formation and rhizosphere colonization of the genetically engineered strain SQR9XYQ can be induced by xylose, and the application of strain SQR9XYQ can improve the biocontrol efficacy to both cucumber and tomato wilt disease. In Figure 7, the current model for the *degQ* gene engineered strain *B. velezensis* SQR9XYQ colonized on the cucumber root surface in the rhizosphere is presented.

DegS-DegU is one of 10 well-characterized systems that regulate several post exponential processes such as the competence for DNA uptake, bacterial motility, biofilm formation, and degradative enzyme synthesis (Murray et al. 2009). Previous research reported that DegU:P is essential for both flagellum formation and biofilm formation. Low levels of DegU:P can activate transcription of the *flgB-sigD* operon, while higher levels of DegU:P activate genes involved in complex colony architecture and biofilm formation (Kobayashi 2007). The most abundant root exudates are sugar and protein, and xylose is a typical carbohydrate secreted from the root (Bacilio et al. 2003), and because the concentration of root exudates including xylose gradually increases from bulk soil to that of the rhizosphere, it is an ideal system to gradually activate the response regulator DegU that controls the serial expression of genes for flagellum formation and biofilm formation by gradually inducing the expression of the *degQ* gene.

Fig. 4. CLSM micrographs of both typical roots and dead roots (cucumber seedlings without stem and leaf) colonized by GFP-tagged strains. The green fluorescence depicts the live strains of *Bacillus velezensis* SQR9 and its derivative strains colonized on the cucumber roots. Dead roots in the hydroponics system were used for images A to H, and typical roots in the hydroponics system were used for I to L. **A**, SQR9 without xylose in the medium. **B**, SQR9 with 0.3% xylose in the medium. **C**, SQR9 with 0.6% xylose in the medium. **D**, SQR9 with 0.9% xylose in the medium. **E**, SQR9XYQ without xylose in the medium. **F**, SQR9XYQ with 0.3% xylose in the medium. **G**, SQR9XYQ with 0.6% xylose in the medium. **H**, SQR9XYQ with 0.9% xylose in the medium. **I**, Control. **J**, $\Delta degQ$ is a *degQ* gene mutant. **K**, Wild-type SQR9. **L**, SQR9XYQ.

As expected, the results indicated that the root colonization ability of the genetically engineering strain is improved compared with the wild-type strain in hydroponic systems. The antibiosis biocontrol activity of *B. velezensis* strains has been demonstrated in different plants and against various pathogens (Xu et al. 2013; Li et al. 2014). The DegS-DegU system activates the production of at least four antibiotics in *B. velezensis* or *B. subtilis* strains including three lipopeptide antibiotics (iturin A, bacillomycin D and fengycin) and one polyketide antibiotic (difficidin) (Xu et al. 2014; Kobayashi 2015). In this study, we showed that the expression of bacillomycin D and difficidin in the root-colonized SQR9XYQ cells is higher than those in the planktonic SQR9XYQ cells. In addition, bacillomycin D contributes to biological control through its involvement in the bacterial colonization on the root and enhancement of the plant immune system.

Plants in the rhizosphere serve as hosts to a large diversity of rhizosphere microbiota, signifying that secretion of root exudates is critical for such plant-microbe interactions (Kamilova et al. 2006). Signaling studies in the rhizosphere have focused on close interactions between plants and symbiotic microorganisms and are now likely to expand to other microorganisms (Venturi and Keel

Fig. 5. The root colonization efficiency and antibiotic expression were improved in SQR9XYQ compared with the wild-type strain SQR9. **A**, The population of SQR9 and SQR9XYQ strains colonized on cucumber seedling roots after 2 and 4 days incubation. **B**, The population of SQR9 and SQR9XYQ strains colonized on tomato seedling roots after 2 and 4 days incubation. **C**, Antibiotic expression of SQR9 and SQR9XYQ strains colonized on cucumber and tomato root at 4 days. The *recA* gene of SQR9 was used as an internal reference gene.

2016). Plant roots actively release organic compounds, which attract root-colonizing bacteria to the rhizosphere (Bulgarelli et al. 2013). L-Malic acid secreted from *Arabidopsis* roots selectively signaled and recruited the beneficial rhizobacterium *B. subtilis* FB117 in a dose-dependent manner, while oxalic acid exhibited significantly reduced efficacy (Rudrappa et al. 2008).

It has identified that the plant polysaccharides pectin, arabinogalactan, and xylan as environmental cues that trigger biofilm formation by plant growth-promoting *B. subtilis* (Beauregard et al. 2013). However, we still know relatively little about the molecular processes underlying the ability of the rhizobacteria to colonize roots. In contrast, much is known about the mechanism of biofilm production *in vitro* by plant growth-promoting strains. To bypass this difficulty, engineering interactions between plants and microbes have been proposed as a solution. For example, the application of the flagellar peptide flg22 or that of the bacterial toxin coronatine to the foliar system of *Arabidopsis* induced the expression of the malic acid transporter ALMT1, leading to an increased malic acid concentration in the rhizosphere, and this increased concentration of malic acid increased the population

of the growth-promoting strains *B. subtilis* FB1A7 by 10-fold, causing an induced systemic resistance response in plants against *P. syringae* pv. *tomato* (Lakshmanan et al. 2012). Similarly, in our study xylose was designed as a signal for DegS-DegU activation, which is critical to elicit antibiosis biocontrol activity in *B. velezensis* and the root colonization process.

Recent research demonstrated that the plant defense signal methyl salicylate elicits antibiosis biocontrol activity from *B. subtilis* 3610 and related bacteria through DegS-DegU activation (Kobayashi 2015). However, the molecular mechanism behind this remains unclear. Whether this genetically engineered strain SQR9XYQ influences the composition of the microbial community in the rhizosphere is an intriguing question for future studies. Although the future potential for applying recombinant bacteria in agriculture is due to the biosafety concern, the knowledge regarding the mechanisms of colonization might lead to research on natural selection of better biocontrol strains.

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Fig. 6. Effects of different treatments on the incidence of both cucumber and tomato wilt disease. Bars with different letters indicate significant differences among the four treatments following Duncan's test ($P < 0.05$). CK (control) potting soil was inoculated with only one pathogen (*Fusarium oxysporum* or *Ralstonia solanacearum*). $\Delta degQ$ potting soil was inoculated with both pathogens (*F. oxysporum* or *R. solanacearum*) and *degQ* mutant strain of *Bacillus velezensis* SQR9. SQR9 potting soil was inoculated with both pathogens (*F. oxysporum* or *R. solanacearum*) and *B. velezensis* SQR9 wild-type strain. SQR9XYQ potting soil was inoculated with both pathogens (*F. oxysporum* or *R. solanacearum*) and *degQ* gene engineered strain *B. velezensis* SQR9XYQ.

Fig. 7. Current model for the *degQ* gene engineered strain *Bacillus velezensis* SQR9XYQ colonized on the cucumber root surface in the rhizosphere. The levels of DegU phosphorylation in the *B. velezensis* SQR9XYQ is increased along a xylose concentration gradient in the rhizosphere.

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