Beneficial Rhizobacterium *Bacillus amyloliquefaciens* SQR9 Induces Plant Salt Tolerance through Spermidine Production

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The inoculation of plants with plant-growth-promoting rhizobacterium has been an effective strategy for enhancing plant salt tolerance to diminish the loss of agricultural productivity caused by salt stress; however, the signal transmitted from bacteria to the plant under salt stress is poorly understood. In this study, the salt tolerance of *Arabidopsis thaliana* and *Zea mays* was enhanced by inoculation with *Bacillus amyloliquefaciens* SQR9. Using dialysis bags with different molecular weight cutoffs, we sorted through the molecules secreted by SQR9 and found that spermidine is responsible for enhancing plant salt tolerance. An SQR9 $\Delta speB$ mutant deficient in spermidine production failed to induce plant salt tolerance. However, the induction of plant salt tolerance was disrupted by mutating genes involved in reduced glutathione (GSH) biosynthesis and the salt overly sensitive pathway in *Arabidopsis*. Using quantitative real-time polymerase chain reaction, this study demonstrated that spermidine produced by SQR9 leads to increased glutamine synthetase and glutathione reductase gene expression, leading to increased levels of GSH, which is critical for scavenging reactive oxygen species. SQR9-derived spermidine also upregulates the expression of *NHX1* and *NHX7*, which sequesters Na^+ into vacuoles and expels Na^+ from the cell, thereby reducing ion toxicity.

Salt stress is one of the most critical agricultural problems (Shabala and Cuin 2008). Excess salinity imposes nutrient deficiency, osmotic stress, oxidative stress, and ion toxicity on plants and, consequently, leads to inhibition of plant growth and even death (Chinnusamy et al. 2006). Therefore, it is necessary to improve plant salt tolerance to enhance agricultural production.

Plant tolerance to salt stress is mainly mediated through hormone regulation, osmotic homeostasis, detoxification, and ionic homeostasis (Deinlein et al. 2014). High salinity disrupts plant phytohormone homeostasis, and hormone regulation is necessary

for controlling plant growth, stomatal closure, and the expression of several phytohormone-dependent genes (Dinnyen et al. 2008; Geng et al. 2013). The accumulation of osmoprotectants reduces water loss, maximizes water uptake, and mitigates osmotic stress induced by salinity (Székely et al. 2008; Upadhyay et al. 2012). Antioxidants can scavenge reactive oxygen species (ROS), which would be overproduced during salt stress and cause plant programmed cell death, thereby protecting the plant from the damage caused by salt stress (Meyer et al. 2007; Mittler and Blumwald 2015; Moschou et al. 2008;). Moreover, some active transporters in the membrane, such as Na^+/H^+ antiporter (NHX) and high-affinity K^+ transporter (HKT), can aid in salt tolerance by transporting Na^+ out of the cytoplasm (Deinlein et al. 2014; Zhu 2001).

Several bacteria that colonize plant roots were found to benefit plants through complex interactions and, therefore, were termed plant-growth-promoting rhizobacteria (PGPR). Plant roots can release specific compounds such as malic acid to attract PGPR (Fan et al. 2012; Rudrappa et al. 2008). Under stress, PGPR can induce chemical and physical changes in the plant to help plants against abiotic stress, called induced systemic tolerance (Dimkpa et al. 2009; Ma et al. 2014; Yang et al. 2009).

Volatile organic compounds (VOC) emitted from *Bacillus subtilis* GB03 have been reported to trigger induced systemic tolerance in plants (Zhang et al. 2008, 2010). VOC emitted from GB03 were able to facilitate shoot to root Na^+ recirculation and reduce Na^+ import in *Arabidopsis* roots by regulating the transcription of the HKT1 in shoots and roots of *Arabidopsis*. Under osmotic stress, phosphoethanolamine N-methyltransferase (PEAMT) expression was upregulated by VOC emitted from GB03, which increased glycine betaine and choline synthesis in *Arabidopsis*, helping the plants tolerate osmotic stress via an abscisic acid (ABA)-independent pathway (Liu and Zhang 2015; Zhang et al. 2010). Other molecules produced by PGPR strains that confer plant tolerance to abiotic stress include 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades the ethylene precursor ACC (Dimkpa et al. 2009; Glick et al. 2007; Mayak et al. 2004); trehalose, which regulates carbon and nitrogen metabolism (Suárez et al. 2008); and indole-3-acetic acid (IAA) and cytokinin, which regulate genes involved in stress tolerance by maintaining plant hormone homeostasis (Dodd and Pérez-Alfocea 2012; Yang et al. 2009). In short, organic compounds produced by PGPR regulate plant photosynthesis, hormone homeostasis, osmotic homeostasis, cell membrane integrity, detoxification, and ionic homeostasis to confer plant salt tolerance.

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The mechanisms allowing plants to tolerate salt stress are very complicated. New mechanisms and genes involved in plant salt tolerance are constantly reported. Thus, other unknown mechanisms involved in PGPR-induced plant salt tolerance may exist. Our previous studies revealed that *B. amyloliquefaciens* SQR9, a well-studied PGPR strain, enhanced plant salt tolerance by regulating phytohormone, osmolyte, antioxidant, and ion content in plants (Chen et al. 2016). In contrast with the known mechanisms by which PGPR induce plant salt tolerance, this study showed that spermidine produced by SQR9 confers plant salt tolerance in the two following ways: by regulating glutamine synthetase (GS) and glutathione reductase (GR) expression to increase reduced glutathione (GSH) levels, thus alleviating ROS damage; and by regulating Na⁺/H⁺ antiporters in the tonoplast (NHX1) and Na⁺/H⁺ antiporters in the plasma membrane (NHX7) expression and activating salt overly sensitive (SOS) proteins to control Na⁺ content in plants.

RESULTS

The molecular weight of the SQR9-secreted signal that confers plant salt tolerance is between 100 and 500 Da.

Under salt stress, SQR9 inoculation increased the number of *Arabidopsis* lateral roots but not primary root length (Supplementary Fig. S1) and enhanced plant growth (Fig. 1). SQR9 inoculation increased photosynthetic rate and reduced ROS (e.g., H₂O₂) content (Supplementary Fig. S2). Previous studies

showed that SQR9 conferred plant salt tolerance by inhibiting the increase of ABA, reducing Na⁺ content, and enhancing total soluble sugar (TSS) and GSH content as well as catalase (CAT) and peroxidase activity in the plant (Chen et al. 2016). However, the signal released by SQR9 that affects plant salt tolerance pathways was not clearly determined. To identify the molecular signal produced by SQR9 and the pathways affected by this signal, SQR9 was wrapped in dialysis bags of different molecular weights and tested for the ability to induce plant salt tolerance. This allowed us to determine the range of the molecular weight of the signal. The fresh weight and height of plants inoculated with SQR9 were higher than plants inoculated with inactive SQR9 (negative control). The biomass of plants treated with SQR9 wrapped in dialysis bags with a molecular weight cut-off (MWCO) greater than or equal to 500 Da was higher than the negative control (Fig. 1A, B, and C). There was no significant difference between plants treated with SQR9 wrapped in 100-Da MWCO dialysis bags and the negative control. The results obtained with *Arabidopsis* were similar to those obtained with maize. We checked the growth of SQR9 in 100- and 500-Da dialysis bags in the rhizosphere and they showed similar growth curves (Supplementary Fig. S3). Leaf chlorophyll was tested as an indicator of plant phenotype, and low leaf chlorophyll content was used as indicator of impaired plants. The trend of leaf chlorophyll content was similar to plant biomass (Fig. 1D). These results indicate that the molecular weight of the SQR9-derived signal that effectively confers plant salt tolerance is between 100 and 500 Da.

Fig. 1. Effects of *Bacillus amyloliquefaciens* SQR9 secretions of different molecular weights on plant salt tolerance. **A**, Representative images of plants treated with SQR9 wrapped in different molecular weight cut-off dialysis bags (5,000, 2,000, 1,000, 500 and 100 Da). **B**, Quantification of maize fresh weight and plant height. **C**, Quantification of *Arabidopsis* fresh weight and plant height. White and black bars represent plant fresh weight and plant height, respectively. **D**, Chlorophyll content (SPAD value) was measured. Salt concentration was 100 mM NaCl. SQR9 treatment = plants inoculated by SQR9 without dialysis bag (positive control); CK = negative control; and LS5000, LS2000, LS1000, LS500, and LS100 = living SQR9 wrapped in 5,000-, 2,000-, 1,000-, 500-, and 100-Da dialysis bags, respectively. White and black bars represent maize and *Arabidopsis*, respectively. Mean values in the same column with different letters indicate significant differences (Duncan's least significant difference, $P \leq 0.05$, $n = 21$ for maize, and $n = 48$ for *Arabidopsis*).

The signal molecule confers plant salt tolerance by regulating GSH and Na⁺ contents.

Our previous study showed that many physiological indexes of plants inoculated with SQR9 are affected, including ABA, TSS, CAT, and peroxidase activity, as well as GSH and Na⁺ content (Chen et al. 2016). To confirm which primary mechanisms for plant salt tolerance were induced by the secreted signal (100 to 500 Da), the physiological indicators of various pathways that could be affected by SQR9 inoculation were measured in the presence or absence of SQR9-secreted molecules between 100 to 500 Da. GSH content in maize and *Arabidopsis* inoculated with SQR9 or SQR9 wrapped in 500-Da dialysis bags was significantly higher than plants treated with inactive SQR9 or SQR9 wrapped in a 100-Da MWCO dialysis bag (Fig. 2A and B). However, ABA content and TSS content in both *Arabidopsis* and maize were not affected by SQR9-secreted molecules between 100 and 500 Da (Supplementary Fig. S4A, B, C, and D). In *Arabidopsis*, CAT activity was higher in plants inoculated with SQR9 wrapped in 500-Da dialysis bags than in that with 100-Da dialysis bags; however, the same experiments in maize did not show significant difference between the 100- and 500-Da treatment. Furthermore, the Na⁺ content in maize and *Arabidopsis* inoculated with SQR9 or SQR9 wrapped in 500-Da MWCO dialysis bags was significantly decreased compared with plants inoculated with inactive SQR9 or SQR9 wrapped in a 100-Da MWCO dialysis bag (Fig. 2C and D). However, the Na⁺ content in a maize shoot (stem and leaf) inoculated with SQR9 or SQR9 wrapped in 100- and 500-Da MWCO dialysis bags was

significantly decreased compared with plants inoculated with inactive SQR9 (Fig. 2C). This result indicated that one or more compounds with a molecular weight smaller than 100 Da were involved in reducing Na⁺ content in shoots. These results revealed that an SQR9-secreted molecule with a molecular weight between 100 and 500 Da confers plant salt tolerance by enhancing the GSH content in plants, reducing the Na⁺ content in *Arabidopsis* and roots of maize, and enhancing CAT activity in *Arabidopsis*.

Verification of the major plant salt tolerance pathway induced by SQR9.

To confirm the roles of CAT, GSH, and ion homeostasis in SQR9-induced plant salt tolerance, *Arabidopsis* mutants deficient in CAT, the GSH metabolic pathway, and the SOS pathway were inoculated with SQR9. The *cat2-1* mutation plant deficient in CAT activity showed salt tolerance responding to SQR9 inoculation as well as the wild-type plant (Supplementary Fig. S5). The *gs* and *gr* mutations deficient in glutamate-cysteine ligase and GR, respectively, were sensitive to salt stress in the presence of SQR9, indicating that reduced GSH is involved in SQR9-dependent salt stress tolerance (Fig. 3; Supplementary Fig. S6). Mutation in *hkt1*, which is involved in Na⁺ circulation from shoot to root and Na⁺ transportation into the plant cell, did not alter the response of plants to SQR9 under salt stress (Fig. 3). *NHX1* and *NHX7* (*SOS1*) encode Na⁺ transporters located at the vacuolar membrane and plasma membrane, respectively, and control the Na⁺ dynamic in plant cells. Deletion of either of these

Fig. 2. Effects of *Bacillus amyloliquefaciens* SQR9 secretions of different molecular weights on reduced glutathione (GSH) content and Na⁺ content of plants. **A**, GSH content and **C**, Na⁺ content in maize were measured after maize was treated with *B. amyloliquefaciens* SQR9 secretions of different molecular weights under salt stress for 20 days. **B**, GSH content and **D**, Na⁺ content were measured in *Arabidopsis* after being similarly treated for 14 days. Columns: 100 mM CK = plants treated with inactivated SQR9 under 100 mM NaCl (negative control) and 100 mM SQR9 = plants inoculated with SQR9 under 100 mM NaCl (positive control). Different letters indicate significant differences between treatments (Duncan's least significant difference test, $P \leq 0.05$, $n = 5$, mean $\pm$ standard deviation).

two genes resulted in the loss of SQR9-induced plant tolerance to salt stress. In addition, mutation of *sos2* or *sos3*, which aid in regulating the Na⁺ content in the cytoplasm, resulted in the loss of SQR9-induced plant tolerance to salt stress (Fig. 3). The GSH metabolic pathway was related to ROS but not Na⁺ content in plants; thus, the Na⁺ content of *gs* and *gr* mutants was still reduced significantly with inoculation of SQR9. Meanwhile, the Na⁺ content of *hkt1* mutants was reduced by SQR9 significantly, which was similar to that of wild-type plants. However, there was no significant difference between the Na⁺ content of *sos1*, *sos2*, *sos3*, and *nhx1* mutants inoculated with SQR9 and the control under salinity (Supplementary Fig. S7). These results showed that GSH synthesis (controlled by GS and GR) and the SOS pathway (controlled by NHX1, SOS1, SOS2, and SOS3) are necessary for SQR9 to trigger plant salt tolerance.

The SQR9-produced signal that confers plant salt tolerance is spermidine.

To identify the signal responsible for conferring plants tolerance to salt stress, the 100- to 500-Da SQR9 secretions were extracted with different organic solvents. Under salt stress, the plant fresh weight increased when treated with SQR9 secretions from the aqueous, ethyl ether, and especially chloroform extracts (Fig. 4). However, the root fresh weight had no significant difference and even significantly decreased when treated with the SQR9 secretion extracted with ethyl acetate and n-pentane, respectively (Fig. 4). These results reveal that the key signal molecule displayed the highest solubility in chloroform phase but was insoluble in ethyl acetate and n-pentane. The SQR9-derived molecules between the molecular weights of 100 and 500 Da were classified. Based on the knowledge of chemicals with activity to affect plant physiology, IAA, gibberellin acid 3 (GA3), transzeatin (ZT), trehalose, and some types of polyamines (PA) were selected as potential signals with a molecular weight between 100 and 500. These potential signals were tested for solubility in the aforementioned extraction reagents. IAA, GA3, ZT, and trehalose are poorly soluble in chloroform. However, PA displayed the greatest solubility in chloroform compared with the other reagents; as a consequence, they are considered to be potential signals secreted by SQR9 for conferring plant salt tolerance. PA produced by

SQR9 were detected by high-performance liquid chromatography (HPLC). Spermidine was detected in both extracellular and intracellular samples of SQR9, and the content was 26.4 and 29.9 μ M, respectively, in the bacterial culture (optical density at 600 nm [OD₆₀₀] = 1), whereas spermine was not detected (Fig. 5A, B, and C). The *B. amyloliquefaciens* SQR9 genome was searched for genes related to PA synthesis, and *speB* was found to be the key gene in PA biosynthesis (Fig. 5D). The *speB* gene was knocked out, and the mutant strain was complemented with the entire *speB* gene. The mutation of *speB* caused a deficit in extra- and intracellular spermidine production. The extracellular and intracellular spermidine contents for mutant-complemented strain Δ *speB* (*c- Δ speB*) were 22.2 and 26.6 μ M, respectively, in the bacterial culture (OD₆₀₀ = 1) (Fig. 5C). There was no significant difference between the growth rate of Δ *speB*, *c- Δ speB*, and wild-type SQR9 strains in either Luria-Bertani (LB) medium or Murashige and Skoog (MS) medium (with or without 100 mM NaCl)

Fig. 4. Effects of various SQR9 secretion extracts on plant biomass under salt stress. SQR9 secretions and Landy medium (control) were extracted with aqueous, ethyl acetate, ethyl ether, n-pentane, and chloroform phases. Values indicate the changes induced by SQR9 secretions extracted with different phases compared with the Landy medium control. Asterisks indicate statistically significant differences between the control and plants treated with SQR9 secretions (*t* test, *n* = 3, mean $\pm$ standard deviation); * and ** indicate *P* $\leq$ 0.05 and 0.01, respectively.

Fig. 3. Effects of *Bacillus amyloliquefaciens* SQR9 on the response of *Arabidopsis* mutants to salt stress. The first five mutants (*sos1*, *sos2*, *sos3*, *nhx1*, and *hkt1*) are missing genes related to ion balance in the plant. The last two mutants (*gr* and *gs*) are involved in reduced glutathione synthesis. Different *Arabidopsis* mutants were inoculated with SQR9 or heat-inactivated SQR9 as a control. **A**, Plant shoot fresh weight and **B**, root fresh weight were measured under salt stress for 14 days. Black and white bars represent SQR9 and heat-inactivated SQR9 control treatments, respectively. Values are the mean $\pm$ standard deviation from 48 replicates. Columns: col = wild-type *Arabidopsis*; *sos1*, *sos2*, and *sos3* mutants were treated with 50 mM NaCl; and *nhx1*, *hkt1*, *gr*, and *gs* mutants were treated with 100 mM NaCl. Asterisks indicate statistically significant differences between plants inoculated with SQR9 and control plants (*t* test); * and ** indicate *P* $\leq$ 0.05 and 0.01, respectively.

(Supplementary Fig. S8). These results revealed that *speB* gene knock out did not affect SQR9 growth.

SQR9-produced spermidine induced plant salt tolerance.

To study the role of spermidine in inducing plant salt tolerance, plants growing on plates were treated with different concentrations of spermidine under salt stress. The optimal concentration of spermidine that effectively enhances plant salt tolerance is between 1 and 10 μM (Fig. 6A). The *speB* gene was the key gene of synthesis of putrescine and spermidine (Fig. 5D). To determine whether spermidine was the key SQR9 signal, dicyclohexylamine (DCHA), an inhibitor of spermidine synthase, was used to inhibit the production of spermidine by SQR9 (Fig. 6B) (Biondi et al. 1988). The addition of DCHA resulted in an inability of SQR9 to confer plant salt tolerance (Fig. 6C). Furthermore, the mutant strain $\Delta\textit{speB}$ did not aid plants against salt stress but promoted *Arabidopsis* growth in the absence of salt stress (Fig. 6D; Supplementary Fig. S9). As expected, complementation strain *c-ΔspeB* conferred the same level of plant salt tolerance as the wild-type SQR9 (Fig. 6E). Moreover, the deficiency of the *speB* mutant strain in conferring plant salt tolerance was rescued by purified spermidine (5 μM) (Supplementary Fig. S10). These results indicate that SQR9-produced spermidine plays a vital role in inducing plant salt tolerance.

Spermidine confers plant salt tolerance through regulating GSH and Na^+ contents.

We found that the 100- to 500-Da compounds secreted by SQR9 affected plant GSH and SOS pathway to induce plant salt tolerance in this study; therefore, we wanted to check whether spermidine functioned through regulating GSH and Na^+ content. Transcription levels of *GS*, *GR*, *NHX1*, and *NHX7* were examined in *Arabidopsis* upon inoculation with or addition of SQR9, a spermidine-production-deficient mutant, the complementary strains, or pure spermidine. The *GS* and *GR* genes encode key enzymes involved in GSH biosynthesis and reduction pathways. *NHX1* and *NHX7* are the key genes involved in reducing

Na^+ content in the cytoplasm. Transcription of *GS*, *GR*, *NHX1*, and *NHX7* in *Arabidopsis* was upregulated upon treatment with either pure spermidine or upon inoculation with wild-type SQR9 compared with plants inoculated with inactive SQR9 (Fig. 7). Inoculation with the mutant strain $\Delta\textit{speB}$ did not lead to a significant increase in *GS*, *GR*, *NHX1*, and *NHX7* transcription. These results indicate that SQR9-produced spermidine mainly regulated the expression of *GS* and *GR* genes which, in turn, enhanced the GSH content in plants. These data further suggest that enhanced transcription of *NHX1* and *NHX7* by SQR9-derived spermidine might be involved in the mechanism by which SQR9 protects plants against salt stress.

To ensure the independence of spermidine functions on other pathways such as HKT1, the responses of the mutant plants to spermidine were investigated. Results showed that spermidine induced *hkt1* mutant plant tolerance to excess salt; however, spermidine could no longer induce the tolerance of plants deficient in *SOS1* *SOS2*, *SOS3*, *NHX1*, *GR*, or *GS* (Fig. 8). These results are consistent with the influence of SQR9 on *Arabidopsis* mutants and confirm that spermidine induces plant salt tolerance through the SOS pathway and GSH metabolism (Figs. 3 and 8).

DISCUSSION

Our previous study has demonstrated that SQR9 maintains photosynthesis, reduces Na^+ content, enhances antioxidant and TSS content, as well as inhibits ABA accumulation in plants to help plants tolerate salt stress (Chen et al. 2016). In this research, we identified the major signal produced and secreted by SQR9, which conferred plant salt tolerance, as spermidine. Moreover, we found that spermidine conferring plant tolerance to salt stress is dependent on regulating the SOS pathway and GSH synthesis, whereas CAT, TSS, and ABA in *Arabidopsis* and maize are not necessary for the induced tolerance to salt stress by SQR9-produced spermidine. However, the SOS pathway and GSH synthesis are both necessary for SQR9-induced plant salt tolerance. In the present study, SQR9-produced spermidine was

Fig. 5. High-performance liquid chromatography (HPLC) analysis of **A**, extracellular and **B**, intracellular spermidine from *Bacillus amyloliquefaciens* SQR9, $\Delta\textit{speB}$, and *c-ΔspeB* strains. **C**, Extracellular and intracellular spermidine content from SQR9, $\Delta\textit{speB}$, and *c-ΔspeB* strains was quantified. **D**, Polyamine biosynthetic pathway in *B. amyloliquefaciens* SQR9. Values indicate the mean $\pm$ standard error from three replicates. Different letters above the columns indicate statistically significant differences (Duncan's least significant difference test, $P \leq 0.05$).

Fig. 6. A, Plant salt tolerance was affected by different concentrations of synthetic spermidine. Salt concentration is 100 mM NaCl. CK = negative control. B, Spermidine concentration of culture filtrates of wild-type SQR9 and SQR9 treated with dicyclohexylamine (DCHA). C, Effect of DCHA, an inhibitor of spermidine synthase, on plant salt tolerance. The fresh weight of *Arabidopsis* was measured after 14 days of treatment under salt stress. The fresh weight of *Arabidopsis*, treated with *Bacillus amyloliquefaciens* SQR9, Δ speB, and c- Δ speB strains and the heat-inactivated SQR9 control, was measured under D, nonstress and E, salt stress conditions. White and black bars represent shoot and root fresh weights, respectively. Multiple statistical analyses were performed in groups labeled with the same uppercase letter. Different lowercase letters above the columns indicate statistically significant differences (Duncan's least significant difference test, $P \leq 0.05$, $n = 48$).

shown to be the major signal that induced plant salt tolerance (Fig. 6E). The bacterially produced spermidine may induce systemic tolerance to salt stress by regulating the GSH content and Na^+ dynamics at the transcriptional level in plants. Overproduction of ROS induced by salt stress serves as a signal to regulate Na^+ homeostasis and causes damage to plants (Deinlein et al. 2014; Moschou et al. 2008). We suppose that the excess ROS caused by salt stress would not be reduced at the early stage of inoculation of SQR9. After salt stress for 14 days, ROS content would be reduced by the bacterial-produced spermidine due to the accumulation of GSH (Figs. 2 and 8).

Furthermore, spermidine regulates expression of NHX1 (the Na^+ transporters located at the vacuolar membrane) and SOS proteins to sequester Na^+ into vacuoles and expel Na^+ from the cell, thereby alleviating Na^+ toxicity (Figs. 2, 8, and 9). In the present study, the Na^+ contents of *sos* mutant plants were relatively higher compared with other reports (24 h) due to the longer treatment time with salt (14 days) (Zhang et al. 2008; Zhu et al. 1998). Zhu et al. (1998) reported that Na^+ content in *sos1* is significantly lower than that in *sos2* and *sos3* in the presence of salt stress but we did not detect the significant difference. The reason for the inconsistency might be the prolonged treatment time with salt, leading to damage of *sos1* (Shi et al. 2002). It is reported that SOS3 is necessary for the SOS pathway in roots but not in shoots (Deinlein et al. 2014; Quan et al. 2007). However, we found that both roots and shoots of *sos3* showed deficiency in response to SQR9 under salt stress. It is believed that roots of *sos3* would be damaged by salt stress in the presence of SQR9 and, consequently, the shoot would be affected by the impaired root. That would be the reason why the decrease of shoot biomass and increase of Na^+ content in shoots of *sos3* could not be released by inoculation of SQR9.

Studies of the induction of systemic tolerance by *B. subtilis* GB03 showed that VOC emitted by GB03, such as 2,3-butanediol and acetoin (Farag et al. 2013), are the major compounds that help plants to tolerate abiotic stress (Zhang et al. 2008). Interestingly, the signal transduction conferring plant tolerance in the interaction between *B. amyloliquefaciens* SQR9 and plants is quite different from that in the *B. subtilis* GB03–plant interaction, because VOC emitted by SQR9 did not significantly improve plant salt tolerance (Supplementary Fig. S11). The VOC emitted from GB03 reduce Na^+ content in plants under salt stress by affecting the transcription of HKT1, a K^+ transporter located in the plasma membrane of *Arabidopsis* that regulates Na^+ uptake and shoot-to-root Na^+ recirculation in *Arabidopsis* (Zhang et al. 2008). In addition, SOS3, the protein involved in the regulation of several ion transporters that respond to salt stress, is not required for the GB03 to induce plant tolerance of salt stress (Zhang et al. 2008). In contrast, we found that HKT1 is not required for the spermidine-induced *Arabidopsis* tolerance to salt stress. However, the NHX1 and SOS proteins are essential for exerting the function of SQR9-produced spermidine on plant salt tolerance. It is known that osmotic stress and ion toxicity are always exhibited in plants under salinity conditions (Deinlein et al. 2014; Zhu 2001). GB03 upregulated the expression of *PEAMT* to enhance choline and glycine betaine content, thereby helping *Arabidopsis* tolerate osmotic stress (Zhang et al. 2010). However, SQR9 does not upregulate *PEAMT* expression (data not shown).

In plants, the PA protect plants from salt stress, especially spermidine and spermine. The PA metabolic route interacts with several metabolisms such as biosynthesis of GSH, nitrogen metabolism, the SOS signal pathway, and others (Alcázar et al. 2010; Kusano et al. 2007, 2008). Previous studies have revealed that PA play a critical role in plant tolerance to abiotic stress (Takahashi and Kakehi 2010; Yamaguchi et al. 2006). *Arabidopsis* double-knockout mutant plants (*acl5/spms*), which could no longer produce spermine are highly sensitive to salt,

whereas exogenous synthetic spermine mitigates plant salt hypersensitivity (Yamaguchi et al. 2006). TSP-16, the spermidine synthase overexpressed transgenic plants, showed upregulated expression of various genes related to stress and enhanced plant salt tolerance (Kasukabe et al. 2004).

Fig. 7. Real-time quantitative polymerase chain reaction analysis of *GS*, *GR*, *NHX1*, and *NHX7* transcript levels in *Arabidopsis* in response to *Bacillus amyloliquefaciens* SQR9, ΔspeB , and $c\text{-}\Delta\text{speB}$ strains, and 5 μM spermidine (spd) under salt stress with 100 mM NaCl. CK and 0-CK = plants in response to inactive SQR9 under salt stress and in the absence of salt stress, respectively. Transcription levels were measured after plants were treated for 1 day under salt stress and compared with the control. Different letters indicate significant differences between treatments (*t* test, $n = 3$, $P \leq 0.05$).

Fig. 8. Effects of 5 μM spermidine (spd) on the response of *Arabidopsis* mutants to salt tolerance. **A**, Shoot fresh weight and **B**, root fresh weight of *Arabidopsis* were tested under salt stress for 14 days. White and black bars represent negative control and spermidine (spd) treatments, respectively. Columns: col-50 and col-100 = the wild type treated with 50 and 100 mM NaCl, respectively; *sos1*, *sos2*, and *sos3* mutants were treated with 50 mM NaCl; and *nhx1*, *hkt1*, *gr*, and *gs* mutants were treated with 100 mM NaCl. Values are the mean $\pm$ standard deviation from 48 replicates. An asterisk (*) indicates statistically significant differences between control plants and plants treated with spd (*t* test, $P \leq 0.05$).

Spermidine can be synthesized by several *Bacillus* spp. (Burrell et al. 2010; Sekowska et al. 1998; Xie et al. 2014). *B. subtilis* OKB105 produces spermidine as a growth-promoting substance and promotes *Nicotiana tabacum* growth by regulating expansin expression and reducing ethylene content in plants (Xie et al. 2014). Xie et al. (2014) believe that the growth promotion of *N. tabacum* by OKB105 is achieved by the fact that bacterial spermidine enhanced the biofilm formation of OKB105, thereby enhancing the bacterial ecological fitness. The new finding of this study is that bacterially produced spermidine is more than a growth-promoting substance: it is also a critical molecule that induces maize and *Arabidopsis* salt tolerance (Fig. 6E).

In conclusion, this study demonstrates that spermidine produced by SQR9 is the key molecule that induces plant salt tolerance; accumulation of GSH and regulation of the SOS pathway are required during this process (Fig. 9). As a consequence of SOS regulation, Na^+ is sequestered into vacuoles and expelled from the cell to reduce Na^+ toxicity. In addition, GSH content is enhanced to reduce ROS damage during salt stress. However, the mechanism by which plants sense SQR9-produced spermidine is still not clear, and these questions merit further investigation in future studies.

MATERIALS AND METHODS

Strains and plant culture conditions.

B. amyloliquefaciens SQR9 (CGMCC accession number 5808; China General Microbiology Culture Collection Center) was cultured at 30°C with shaking at 170 rpm for 10 h in LB medium (yeast extract at 5 g liter⁻¹, tryptone at 10 g liter⁻¹, and NaCl at 10 g liter⁻¹; pH 7.0 to 7.2). One culture was adequately hyperthermia inactivated twice and used as a “dead cell” negative control. The cultures were centrifuged at 6,000 rpm for 10 min. The cells were suspended and diluted with double-distilled water to a final concentration of 5×10^6 CFU ml⁻¹.

Maize (*Zea mays* ‘Jingtian’) seed were sterilized and germinated in a growth chamber. Three-day-old seedlings were transplanted into sterilized one-quarter-strength (1/4) Hoagland medium (Hoagland and Arnon 1938) and grown at 25°C in a growth chamber with a photoperiod of 16 h of light and 8 h of darkness. The medium was shaken at 50 rpm for 2 h every day and replaced every 2 days (Chen et al. 2016).

Arabidopsis thaliana (col-0) seed were surface sterilized and then placed on one-half-strength (1/2) MS media (pH 5.7) containing 1.5% sucrose and 0.8% agar (Murashige and Skoog 1962). T-DNA insertion mutants were obtained from The *Arabidopsis* Information Resource (TAIR; <http://www.arabidopsis.org>) and identified in the SALK and GABI-KAT databases. The mutants used in this study were as follows: *gs*, *gr*, *nhx1*, *sos1*, *sos2*, *sos3*,

and *hkt1* (Table 1). *Arabidopsis* mutant seed from the T₂ progeny were sterilized and germinated as described above. Gene-specific primer pairs and T-DNA-specific primers were used for polymerase chain reaction (PCR) screening to identify T-DNA insertions.

Inoculation of strains.

Arabidopsis seed were vernalized at 4°C for 2 days in the dark and then incubated at 22°C in a growth chamber with a photoperiod of 16 h of light and 8 h of darkness. Seven-day-old seedlings were transplanted to 1/2 MS solid medium with or without 100 mM NaCl. To measure the impact of SQR9 on plant tolerance, each plate was inoculated with 2 µl of active SQR9 or dead cell suspension. The *nhx1*, *sos1*, *sos2*, and *sos3* mutants were transplanted onto medium with or without 50 mM NaCl. Ten plates were included in each treatment and six plants were included in each plate to control for variability. In each plate, the left three plants were mutants and the right three plants were the wild type.

Ten-day-old *Arabidopsis* seedlings were detached from 1/2 MS agar plates and transplanted onto 1/4 MS liquid medium. Thirteen-day-old *Arabidopsis* and maize seedlings were treated with or without 100 mM NaCl and inoculated with bacterial suspensions in different MWCO dialysis bags (100, 500, 1,000, 2,000, or 5,000 MWCO). In this experiment, bacteria cells were suspended with 1/4 MS medium and 1/4 Hoagland medium, respectively. The media were shaken at 50 rpm for 2 h every day and replaced every 2 days. Bacterial suspension also was changed every 2 days. The experiments were repeated three times. For each treatment, 48 *Arabidopsis* and 21 maize seedlings were included as replicates.

Determination of plant biomass.

The plant fresh weight was measured by an analytical scale. Plant height and root length were measured with a ruler. Lateral roots were observed and counted with a stereomicroscope.

Determination of leaf chlorophyll, reduced GSH, and Na^+ content.

Chlorophyll content (SPAD value) was measured using a chlorophyll meter (SPAD-502) (Ling et al. 2011). The leaves from different plants used for detection were in the same location on plants. For each treatment, 10 independent samples were measured.

The enzymatic recycling method used to measure GSH was previously described (Airaki et al. 2011; Griffith 1980).

To determine Na^+ content, whole plants were washed with deionized water and dried at 70°C for 2 days. All plant samples were homogenized and filtered using a filter with a 1-mm pore

Fig. 9. *Bacillus amyloliquefaciens* SQR9 induces systemic tolerance to salt stress in plants. Spermidine produced by SQR9 enhances reduced glutathione (GSH) content, thus reducing reactive oxygen species (ROS) content, and regulates the salt overly sensitive (SOS) pathway to sequester Na^+ into vacuoles and expel Na^+ from the cytoplasm to confer plant salt tolerance.

Table 1. *Arabidopsis* mutants used in this study

Mutants	Stock name	Locus	Function
<i>gs</i>	SALK_081530C	At4g23100	Encodes the enzyme glutamate-cysteine ligase
<i>gr</i>	SALK_104319C	At3g24170	Encodes glutathione-disulfide reductase
<i>nhx1</i>	SALK_034001C	At5g27150	Encodes Na^+/H^+ antiporters in the tonoplast
<i>sos1</i>	SALK_092851C	At2g01980	Encodes Na^+/H^+ antiporters in the plasma membrane, SOS1
<i>sos2</i>	SALK_000367C	At5g01820	Encodes protein kinase, SOS2
<i>sos3</i>	SALK_110426C	At4g17615	Encodes calcineurin B-like protein, SOS3
<i>hkt1</i>	CS476306	At4g10310	Encodes high-affinity K^+ transporter 1
<i>cat2-1</i>	SALK_076998	AT4G35090	Encodes catalase 2

size. The Na⁺ in the plants was extracted as described by Zhang and quantified using an inductively coupled plasma spectrophotograph (Zhang et al. 2008).

Fractionation of bacterial secretions.

SQR9 was cultured in Landy medium (Landy et al. 1948) at 30°C for 36 h. Filtered SQR9 secretions and Landy medium (negative control) were freeze dried and then dissolved in 50 ml of sterile distilled water. Then, the 100- to 500-Da SQR9 secretions and Landy medium were collected using 100- and 500-Da MWCO dialysis bags. The 100- to 500-Da SQR9 secretions (50 ml) and Landy medium were partitioned with 50 ml of ethyl acetate and the ethyl acetate phase was separated. Then, the aqueous phase was fractionated with 50 ml of ethyl ether and the ethyl ether phase was separated. The aqueous phase was fractionated with 50 ml of n-pentane and the n-pentane phase was separated. Finally, the aqueous phase was fractionated with 50 ml of chloroform and the chloroform and aqueous phases were separated. Each organic phase was dried under nitrogen gas. This process resulted in five types of fractions: ethyl acetate, ethyl ether, n-pentane, chloroform, and aqueous fractions. The SQR9 secretion without fractionation (total secretion sample) was also kept.

Construction of a *B. amyloliquefaciens* SQR9 mutant strain.

To confirm the contribution of SQR9-derived spermidine to plant salt tolerance, the *speB* gene was completely deleted by double cross-over (Yan et al. 2008; Zhou et al. 2017). The sequences of erythromycin were obtained from pAX01 plasmid. Two partial sequence fragments of the *speB* gene were amplified from SQR9 DNA. Then, the recombinant fragments were fused and transformed into the SQR9 strain to generate the Δ *speB* mutation. To complement the Δ *speB* mutant, the entire *speB* gene was amplified from *B. amyloliquefaciens* SQR9 chromosomal DNA and ligated into the pNW33 plasmid to obtain pNW33-*speB*, which was transformed into the Δ *speB* mutant to get the complementary strain of mutant Δ *speB* (c- Δ *speB*). The primers used to construct these mutants are shown in Supplementary Table S1.

Measurement of spermidine.

The bacterial strains were cultured in Landy medium at 30°C for 36 h. The fermented cultures were centrifuged at 6,000 rpm for 10 min at 4°C. The bacterial cells were treated with lysozyme and suspended in 0.1 M HCl, then sonicated. Pure perchloric acid was added to culture filtrates or lysates to obtain a 5% (wt/vol) solution for 1 h at 4°C. The samples were centrifuged at 10,000 rpm for 30 min. Benzoyl chloride (70 μ l) was added to 5 ml of supernatant. Then, each sample was neutralized with 10 ml of 2 M NaOH, vortexed for 20 s, and incubated at 37°C for 30 min. A saturated NaCl solution and ethyl ether (15 ml) were added to each sample. The organic phase was dried using nitrogen and resuspended in 200 μ l of methanol. The sample was detected by an HPLC system equipped with a UV detector set at 254 nm and a C18 column (Waters). The mobile phase was 60% methanol, the flow rate was 0.7 ml min⁻¹ and the column temperature was 30°C.

To detect the role of spermidine in plant salt tolerance, different concentrations of exogenous spermidine were added to MS medium with 100 mM NaCl, and 10 mM DCHA (an inhibitor of PA biosynthesis) was added to the bacterial growth medium. Each treatment had six plates containing six plants each.

Transcription analysis.

RNA was extracted from plants after 1 day of treatment. Plant tissue was flash frozen in liquid nitrogen and then extracted

using the Qiagen RNeasy plant mini kit (Qiagen). The transcript levels were quantified by reverse transcription polymerase chain reaction using the PrimeScript RT reagent Kit (TaKaRa Dalian Biotechnology). Quantitative real-time (qRT)-PCR assays were performed with SYBR Premix EX Taq (TaKaRa Dalian Biotechnology) using an ABI 7500 Cyclor (Applied Biosystems). Transcription levels of *GS* (encoding GS), *GR* (encoding GR), *NHX1* (encoding Na⁺/H⁺ antiporters in the tonoplast), and *NHX7* (encoding Na⁺/H⁺ antiporters in the plasma membrane) were measured using *ACT* (encoding actin) as an internal reference. For each treatment, three biological replicates are included. The specificity of the amplification was verified by melting-curve analysis and agarose gel electrophoresis. The relative transcription levels were calculated using the 2^{- $\Delta\Delta$ CT} method (Livak and Schmittgen 2001).

Statistical analysis.

The data among different treatments were subjected to analysis of variance. Duncan's multiple range tests ($P < 0.05$) was employed to determine differences among means. Statistical significance was analyzed by *t* test and SPSS (version 18.0; SPSS, Inc.).

Accession numbers.

The sequences of genes used in this article can be found in National Center for Biotechnology Information or TAIR data library under the following accession numbers: *Act* (At3g18780), *NHX1* (At5g27150), *NHX7* (At2g01980), *GR* (At3g24170), and *GS* (At5g35630).

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