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REGULAR ARTICLE

Induced growth promotion and higher salt tolerance in the halophyte grass *Puccinellia tenuiflora* by beneficial rhizobacteria

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Abstract

Background and Aims Soil salinization limits conventional agriculture since most food-based plant cultivars require low soil-sodium (Na^+) levels for robust growth. Moreover, modern agricultural practices, especially in arid environments, can exacerbate soil salinization as belowground water sources utilized in irrigation are frequently tainted with salt. While salt tolerance has previously been shown to be augmented in several glycophyte species by the soil bacterium *Bacillus subtilis* (GB03), here we reported that this beneficial rhizobacterium promotes growth and augments higher salt-tolerance in halophyte grass *Puccinellia tenuiflora*.

Methods The optimal *Bacillus subtilis* strain for *P. tenuiflora* was screened. *P. tenuiflora* was grown from seeds with NaCl (0, 100, 200 and 300 mM) for salt treatments with or without inoculation of *B. subtilis* GB03. Growth parameters, chlorophyll content and endogenous Na^+ and K^+ contents were determined at the time of harvest. Seedlings were grown in medium with 0 or 200 mM NaCl, then were harvested to extract total RNA after 48 h of exposure to GB03 VOCs. Semi-quantitative RT-PCR was used to investigate the relative amount of *PtHKT1*;*5*, *PtHKT2*;*1* and *PtSOS1* in *P. tenuiflora* regulated by GB03.

Results The optimal *Bacillus subtilis* strain for *P. tenuiflora* was GB03. GB03 significantly improved shoot and root growth at two, three, four and five weeks after inoculation. Under various salinity stresses, GB03 significantly promoted growth of *P. tenuiflora* seedlings. Na^+ accumulation was reduced with K^+ accumulation unaffected by GB03 exposure. Therefore, GB03 enhanced selective absorption capacity of *P. tenuiflora* for K^+ over Na^+ (SA) from media. Gene expression analysis demonstrated that GB03 up-regulated *PtHKT1*;*5* and *PtSOS1*, but down-regulated *PtHKT2*;*1* expression, specifically in roots when plants are grown under greatly-elevated salt conditions (200 mM NaCl).

Conclusions Our results presented here established that *B. subtilis* GB03 promoted the growth and improved the salt tolerance and the selective absorption capacity for K^+ over Na^+ in the monocotyledonous halophyte *P. tenuiflora* to a higher level. Interestingly, GB03-triggered up-regulation of *PtHKT1*;*5* and *PtSOS1* and down-regulation of *PtHKT2*;*1* in roots reduced Na^+

Shu-Qi Niu and Hui-Ru Li have contributed equally to this work.

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transport from root to shoot as well as Na^+ uptake in roots. This study provides the physiological and molecular evidence that application of selected bacteria to salt-tolerant Monocots can ameliorate deleterious effects of high soil saline toxicity.

Keywords *Puccinellia tenuiflora* · Growth promotion · Inducible-salt tolerance · *Bacillus subtilis* (GB03) · *HKT* · *SOS1*

Introduction

Earth has a salty surface with vast regions being covered by ocean where Na^+ concentration is about 500 mM; in contrast, K^+ concentration is more than 55 times lower (Flowers 2004). Although higher plants are most likely to have evolved from such a concentrated brine environment, soil salinity can be highly toxic, particularly for salt-sensitive glycophytes (Flowers and Colmer 2008; Munns and Tester 2008; Rozema and Flowers 2008; Shabala and Cuin 2008; Kronzucker and Britto 2011; Zhang and Shi 2013; Maathuis et al. 2014; Roy et al. 2014). Salt-induced damage for such glycophytes includes: reduced photosynthesis, oxidative stress, compromised leaf expansion and induced stomata closure, all leading to lower plant biomass and reduced reproductive success (Rahnama et al. 2010; James et al. 2011; Zhang and Shi 2013). High K^+ levels are essential to suppress activities of proteolytic enzymes (caspase-like proteases and endonucleases) and therefore prevent salt stress-induced programmed cell death in root cells (Shabala and Pottosin 2014). However, elevated soil Na^+ induces K^+ deficiency in plants that in turn perturbs K^+-dependent enzymatic reactions and induce deleterious protein-conformation alterations (Mahajan and Tuteja 2005; Chao et al. 2013; Zhang et al. 2013). Since all major agricultural crops are glycophytes (Zhang and Shi 2013). Increasing soil salinization due to modern agricultural practices poses a significant global threat to agricultural productivity (Flowers 2004; Zhang and Shi 2013; Gupta and Huang 2014).

Plants have evolved complex mechanisms to adapt to saline environments including control of Na^+ uptake, Na^+ xylem loading, Na^+ retrieval from the xylem, Na^+ extrusion from roots, Na^+ vacuole compartmentation and Na^+ extracellular secretion (Blumwald et al. 2000; Munns and Tester 2008; Shabala and Cuin 2008; Zhang et al. 2010; Kronzucker and Britto 2011; Yamaguchi et al. 2013; Zhang and Shi 2013; Liu et al. 2015). High-affinity K^+ transporters (HKTs) are members of a large superfamily of transporters in plants, bacteria and fungi that can be divided into two distinct subfamilies according to their Na^+ and K^+ transport properties (Platten et al. 2006; Horie et al. 2009; Zhang et al. 2010; Zamani Babgohari et al. 2014). Subfamily 1 are Na^+-specific transporters and include several biochemically characterized examples including *Arabidopsis thaliana* AtHKT1;1, *Hordeum vulgare* HvHKT1;4, *Oryza sativa* OsHKT1;4, OsHKT1;*5*, *Triticum aestivum* TaHKT1;4, TaHKT1;5 and *T. monococcum* TmHKT1;4 and TmHKT1;5 (Lindsay et al. 2004; Ren et al. 2005; Huang et al. 2006; James et al. 2006; Davenport et al. 2007; Byrt et al. 2007; Huang et al. 2008; James et al. 2011; Ali et al. 2012; Hill et al. 2013; Byrt et al. 2014). Subfamily 2 are K^+-Na^+ co-transporters with examples including TaHKT2;1, OsHKT2;1, *P. tenuiflora* PtHKT2;1 and HvHKT2;1 (Schachtman and Schroeder 1994; Rubio et al. 1995; Gassmann et al. 1996; Horie et al. 2001; Haro et al. 2005; Kader et al. 2006; Horie et al. 2007; Huang et al. 2008; Ardie et al. 2009). It was suggested that HKT2;1 mediates nutritional Na^+ uptake under low saline condition, and controls Na^+ uptake and mediates high-affinity K^+ uptake under higher saline condition in wheat, rice, *P. tenuiflora* and barley (Laurie et al. 2002; Horie et al. 2007; Ardie et al. 2009; Mian et al. 2011). HKT1;5 retrieves Na^+ from the xylem vessels in the root and has an critical role in restricting the transport of Na^+ from the roots to the leaves and maintaining a high K^+/Na^+ ratio in the leaves in rice, *Triticum monococcum* and wheat (Ren et al. 2005; James et al. 2011; Munns et al. 2012; Platten et al. 2013; Byrt et al. 2014). Salt overly sensitive1 (SOS1) is a plasma membrane Na^+/H^+ antiporter that is important for Na^+ efflux at the cellular level under elevated salt conditions and long-distance Na^+ transport from root to shoot to control salt accumulation in plant shoots (Shi et al. 2000; Shi et al. 2002; Shi et al. 2003; Guo et al. 2012; Liu et al. 2015).

Plant growth-promoting rhizobacteria (PGPRs) are naturally occurring soil microorganisms able to colonize roots and stimulate plant growth (de Zelicourta et al. 2013). Therefore, PGPRs are applied to a wide range of agricultural crops for the purpose of growth enhancement and stress tolerance (Kloepper et al. 1999; Myresiotis et al. 2014). *Bacillus subtilis* as an important species among PGPRs can be isolated from many environments, terrestrial and aquatic, and adapt to grow in

diverse conditions within the biosphere (Stein 2005; Earl et al. 2008). *B. subtilis* has been successfully used for several decades and has been shown the remarkable effects by the reduction of soil-borne fungal plant pathogens, the increase in disease resistance and the growth promotion (Kloepper et al. 2004; Zhang et al. 2011; Gao et al. 2013). GB03 is a commercially *B. subtilis* strain, which enhances plant growth, photosynthesis, iron uptake, as well as disease resistance via emission of volatile organic compounds in the model plant *Arabidopsis* (VOCs) (Ryu et al. 2003, 2004; Zhang et al. 2007, 2008b, 2009; Zhang et al. 2010). Indeed GB03 has been shown to trigger differential expression of approximately 600 transcripts in *Arabidopsis* seedlings including genes related to cell wall modifications, primary and secondary metabolism, hormone regulation as well as biotic and abiotic stress tolerance (Zhang et al. 2007). Especially, Zhang et al. (2008a) reported that GB03 conferred salt tolerance via tissue-specific regulation of *AtHKT1;1* in *Arabidopsis*. Recently, we found that GB03 promoted growth and salt tolerance in wheat (Zhang et al. 2014) and white clover (Han et al. 2014).

Puccinellia tenuiflora is a halophytic monocotyledonous species that is widely distributed in the saline–alkali soil in north China, and used as forage as well as for soil improvement (Wang et al. 2002, 2004). X-ray microanalysis revealed that selective transport of K^+ over Na^+ occurs at the endodermis where the Casparian bands exist, which results in high K^+ accumulation in the shoot and large Na^+ retention in the root (Peng et al. 2004). Compared to wheat, *P. tenuiflora* exhibits stronger selective absorption and transport for K^+ over Na^+ and it was proposed that restricting unidirectional Na^+ influx into roots seems likely to contribute to the salt tolerance of *P. tenuiflora* (Wang et al. 2009). It was reported that *Puccinellia ciliata* was able to maintain highly negative membrane potential under saline conditions (−85 mV for 250 mM NaCl treatment) that contribute to better K^+ retention and higher potassium tissue content (Teakle et al. 2013). Most halophytes are dicot, however, most of the economically important crops are monocot. Therefore, clarifying the salt-tolerance regulation of the monocotyledonous halophyte by beneficial rhizobacterium will provide valuable information toward enhancing salt tolerance of itself and cereal crops as well (Peng et al. 2004; Wang et al. 2004, 2007; Wang et al. 2009; Yu et al. 2011; Guo et al. 2012).

Here is shown that elevated salt tolerance as well as enhanced growth occurs in the halophyte grass *P. tenuiflora* with induction by GB03. The high-affinity K^+ transporters HKT1;5 and HKT2;1 as well as the plasma membrane Na^+/H^+ antiporter SOS1 were examined as likely mechanisms for improved Na^+ retrieval from xylem and Na^+ efflux to the soil, and reduced Na^+ uptake in roots.

Materials and methods

Bacterial cultures

Bacillus subtilis (713), *B. subtilis* (GB03) and *B. pumilus* (2808) were streaked onto Luria Broth (LB) agar plates and incubated at 28 °C in the absence of light for 24 h. Bacterial cells were then harvested from LB agar plates, transferred into liquid LB media and cultured at 28 °C with 250 rpm rotation to yield 10^9 colony forming units (CFU) per mL, as determined by optical density and serial dilutions (Zhang et al. 2008a).

Plant materials and treatments

For physiological work, *Puccinellia tenuiflora* seeds were surface sterilized with 2 % NaClO for 2 min followed by 70 % ethanol for 5 min, rinsed with sterile water for five times, and vernalized for 2 days at 4 °C in the absence of light. Seeds were then planted in Magenta boxes (75 × 75 × 100 mm) containing 120 ml of half-strength solid MS (Murashige and Skoog 1962) media with 0.7 % (*w*/*v*) agar and 1.5 % (*w*/*v*) sucrose supplemented without or with NaCl (0, 100, 200, 300 mM) for salt treatment. 20 μL of prepared bacterial suspension culture as bacterial treatments or the same volume of LB liquid media as control was inoculated into a separate glass vial also containing the same media provided a restricted area within the Magenta box. After seed planting and bacterial inoculation, a plastic collar (75 × 75 × 20 mm) was coupled together as pairs to close the box. Then Magenta boxes were placed in a growth room set to a 14−/10-h light/dark cycle, respectively, using metal halide and high pressure sodium lamps with a total light intensity of 600 μmol photons m^{-2} s^{-1}; temperature was set to 21 ± 4 °C and relative humidity 40 ± 10 %.

Plant physiological measurements

At the time of harvest, plants were removed from the media and roots were rinsed with water to remove attached media; roots and shoots were separated and blotted with paper towel. Fresh weight and root length were measured immediately. Then samples were oven-dried at 80 °C for 3 days to obtain dry weight. Leaf chlorophyll content was estimated according to Lichtentthaler (1987). Approximately 10 mg fresh leaf samples were ground thoroughly with 1 mL 80 % acetone in glass grinder (Fisher Scientific) followed by centrifugation at 13,000 g for 5 min at 4 °C. The supernatant was used for spectrometric measurements at wavelengths of 470, 646.8 and 663.2 nm to estimate total chlorophyll content and chlorophyll a/b.

Ion analysis and calculation of SA value

For ion content analysis, plants were harvested four weeks after exposure to GB03 VOCs. Roots were washed twice for 8 min in ice cold 20 mM $CaCl_2$ to exchange cell-wall-bound K^+ and Na^+, and the shoots were rinsed in deionized water to remove surface salts. Roots and shoots were separated and samples were oven dried at 80 °C for 3 days. Dried tissue was extracted with 10 mL of 100 % HNO_3 for 24 h, followed by incubation at 90–100 °C for 2 h. Digested samples were diluted with water 10-fold. Aqueous K^+ and Na^+ were determined by atomic absorption spectrophotometry (Model 6300; Shimadzu Scientific Instruments, Columbia, MD, USA) (Zhang et al. 2008a; Zhang et al. 2014).

The value of selective absorption for K^+ over Na^+ (SA) was estimated according to the following equation as described by Wang et al. (2009). SA = (K^+/Na^+ in whole plant)/(K^+/Na^+ in medium), which indicates roots' ability to absorb K^+ over Na^+.

Semi-quantitative RT-PCR

For investigating the relative amount of *PtHKT1*;*5*, *PtHKT2*;*1* and *PtSOS1* in *P. tenuiflora* regulated by GB03, seedlings were grown in medium with 0 or 200 mM NaCl, then were harvested to extract total RNA after 48 h of exposure to GB03 VOCs. Total RNA was extracted using RNeasy plant mini kit (Qiagen, Valencia, CA). On-column DNase digestion was performed during RNA extraction to exclude DNA contamination. First strand cDNA was synthesized from 5 μg total RNA using MuMLV-RT (Fisher Scientific, Houston, TX, USA). Primers and Semi-quantitative RT-PCR reaction conditions used for investigating the relative amount of *PtHKT1*;*5*, *PtHKT2*;*1* and *PtSOS1* in *P. tenuiflora* regulated by GB03 were listed in Supplemental Table S1. Agarose gel electrophoresis images were taken by Kodak Gel Logic 100 Imaging System (Fisher Scientific, Houston) and quantified by using Image J 1.33u (http://rsb.info.nih.gov/ij/, National Institute of Health, USA).

Statistical analysis

Results of Na^+ influx, net Na^+ and K^+ uptake rate and genes expression levels are presented as means with SE and data analysis was performed by ANOVA using SPSS statistical software (Ver. 13.0, SPSS Inc., Chicago, IL,USA). Duncan's multiple range test was used to detect a difference between means at a significance level of $P < 0.05$ ($n = 8$).

Results

Growth promotion of *P. tenuiflora* exposed to commercial *Bacillus* strains

Initially several commercially-available PGPR strains, *Bacillus subtilis* (713), *B. subtilis* (GB03) and *B. pumilus* (2808), were screened for *P. tenuiflora* growth promotion. To assay for seedling growth, several physical parameters were recorded four weeks after plant-bacterial exposure to PGPR strains 2808, 713 and GB03 (Fig. 1). All the three PGPR strains significantly improved root length by 24 %, 63 % and 97 %, respectively, compared to control (Fig. 1b). Compared to control, strains 713 and GB03 significantly improved shoot fresh weight (Fig. 1c) by 186 % and 225 %, shoot dry weight (Fig. 1d) by 90 % and 346 %, root fresh weight (Fig. 1e) by 194 % and 317 %, and root dry weight (Fig. 1f) by 103 % and 285 %, respectively ($P < 0.05$). Compared to control, only *B. subtilis* strain GB03 significantly improved leaf chlorophyll content of *P. tenuiflora* by 15 % ($P < 0.05$) (Supplemental Fig. S1); however, both 713 and GB03 significantly improved chlorophyll a/b by 26 % and 23 %, respectively ($P < 0.05$) (Supplemental Fig. S1).

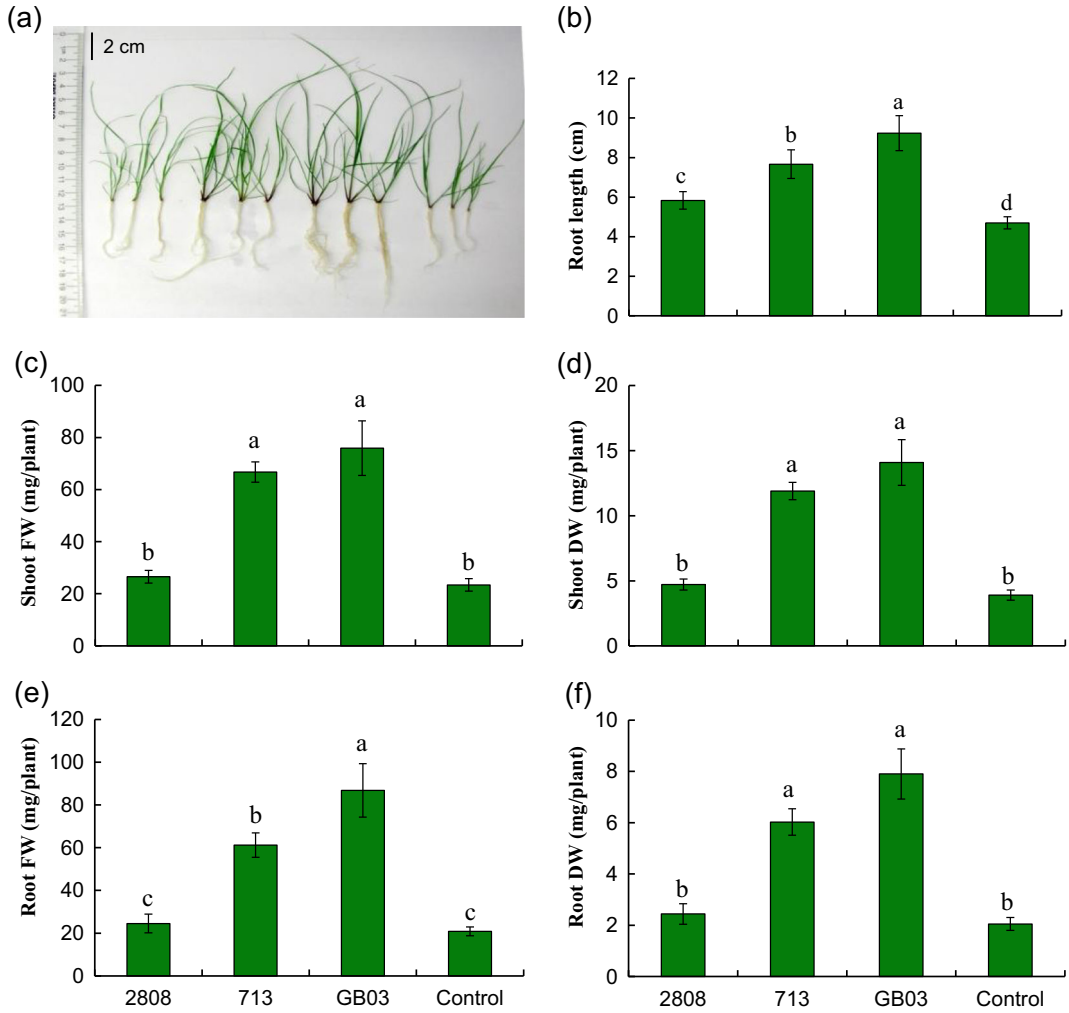

Fig. 1 Effects of *Bacillus subtilis* strains 2808, 713 and GB03 exposure on seedling growth of *P. tenuiflora*, **a** whole plant image, **b** root length, **c** shoot fresh weight (FW), **d** shoot dry weight (DW), **e** root fresh weight and **f** root dry weight. Seedlings were taken image and root length and biomass were measured after four week exposure to *Bacillus subtilis* strains 2808, 713, GB03 and LB medium, respectively. Values are means and bars indicate SDs ($n = 8$). Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test)

Dynamic growth promotion of *P. tenuiflora* by GB03

To test the dynamics of growth promotion to the halophyte grass by *B. subtilis* GB03, seedlings were harvested for measuring biomass two, three, four and five weeks after exposure with GB03. *B. subtilis* GB03 had dynamic growth promotion for *P. tenuiflora* after two weeks of inoculation. GB03 significantly improved shoot fresh weight (Fig. 2a) by 79 %, 154 %, 202 % and 267 %, shoot dry weight (Fig. 2b) by 28 %, 147 %, 233 % and 249 %, root fresh weight (Fig. 2c) by 67 %, 361 %, 250 % and 313 %, and root dry weight (Fig. 2d) by 78 %, 247 %, 264 % and 238 % at two, three, four and five weeks after inoculation, respectively ($P < 0.01$), compared to corresponding controls.

Induced higher salt tolerance of *P. tenuiflora* by GB03

To test whether GB03 inoculation could enhance salt tolerance of the halophyte *P. tenuiflora* further, seedlings were harvested for measuring biomass and ion contents four weeks after GB03 exposure and salt treatments. Seedling growth was remarkably restrained by elevated salinity; however, GB03 significantly promoted growth of *P. tenuiflora* seedling (Supplemental Fig. S2). GB03 improved seedling biomass of *P. tenuiflora*

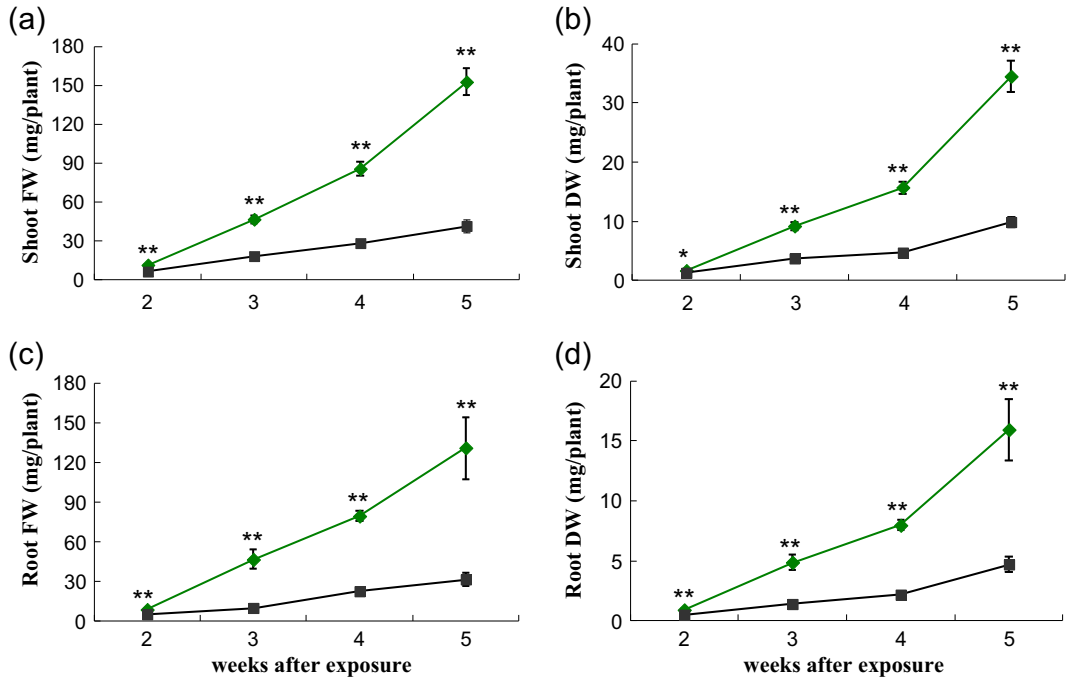

Fig. 2 Dynamic growth promotion of *Bacillus subtilis* strain GB03 exposure in *P. tenuiflora*, **a** shoot fresh weight, **b** shoot dry weight, **c** root fresh weight and **d** root dry weight. Seedling biomass was measured after two, three, four and five week exposure to *Bacillus subtilis* strain GB03. The green and black line represent GB03 exposure and control, respectively. Values are means and bars indicate SDs (n = 8). One asterisk (*) and two asterisks (**) indicate $P < 0.05$ and $P < 0.01$ (t test), respectively, for treatment versus controls

under various salinity stresses. Compared to corresponding control, GB03 significantly improved shoot fresh weight (Fig. 3a) by 202 %, 301 %, 173 % and 63 %, shoot dry weight (Fig. 3b) by 233 %, 316 %, 211 % and 102 %, root fresh weight (Fig. 3c) by 250 %, 79 %, 604 % and 120 %, and root dry weight (Fig. 3d) by 264 %, 101 %, 413 % and 143 % under 0, 100, 200 and 300 mM NaCl treatments, respectively ($P < 0.01$).

To test whether GB03 exposure led to reduced Na^+ accumulation in plants, Na^+ and K^+ contents were assayed in seedlings of *P. tenuiflora* grown under various salinity conditions. Na^+ accumulation was reduced with K^+ accumulation unaffected by GB03 exposure. Compared to corresponding control, GB03 significantly reduced shoot Na^+ content (Fig. 4b) by 43 %, 57 %, 61 % and 60 %, root Na^+ content (Fig. 4e) by 35 %, 86 %, 81 %, and 77 % and total Na^+ content in the whole plants (Fig. 4h) by 39 %, 105 %, 71 % and 72 % with 0, 100, 200 and 300 mM NaCl treatments, respectively ($P < 0.05$). Therefore, K^+/Na^+ ratio was significantly enhanced under various salinity conditions. Compared to corresponding control, GB03 significantly improved shoot K^+/Na^+ ratio (Fig. 4c) by 31 %, 56 %, 60 % and 75 %, root K^+/Na^+ ratio (Fig. 4f) by 6 %, 77 %, 53 % and 78 %, and total K^+/Na^+ ratio in the whole plants (Fig. 6i) by 27 %, 95 %, 68 % and 71 %, respectively ($P < 0.05$), with 0, 100, 200 and 300 mM NaCl treatments, respectively, except for root K^+/Na^+ ratio at non-salinity condition.

To test whether GB03 enhanced selective absorption capacity for K^+ over Na^+ (SA) from media in *P. tenuiflora*, SA value was calculated according to Wang et al. (2009). Figure 5 indicated that GB03 remarkably enhanced SA value of *P. tenuiflora* seedlings. SA value was improved significantly by 95 %, 68 % and 71 % with 100, 200 and 300 mM NaCl treatments, compared to the parallel controls.

Transcriptional regulation of ion transporters by GB03

To probe the molecular basis of GB03-mediated endogenous-Na^+ reductions in both shoot and root tissues, tissue-specific gene regulation of sodium-specific ion transporters, PtHKT1;5, PtHKT2;1 and PtSOS1, were assayed with GB03 exposed seedlings in the media supplemented with 0 or 200 mM NaCl. The expression

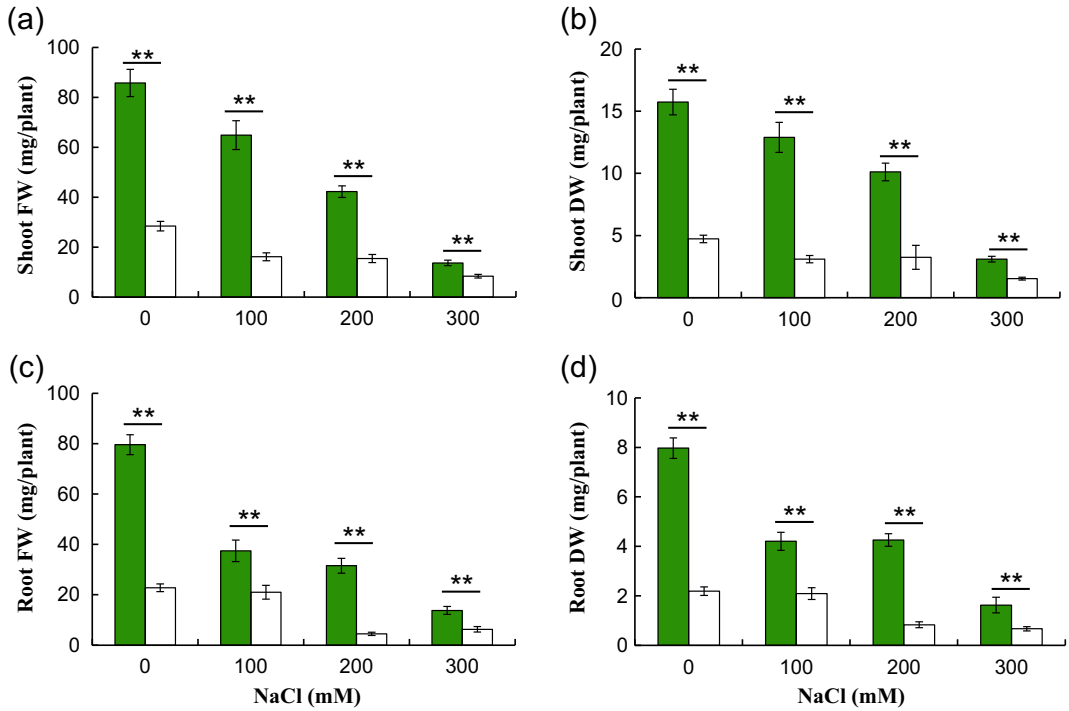

Fig. 3 Effects of *B. subtilis* strain GB03 exposure on salt tolerance of *P. tenuiflora*, **a** shoot fresh weight, **b** shoot dry weight, **c** root fresh weight, and **d** root dry weight. Seedlings were taken image and biomass was measured after four week exposure to *Bacillus subtilis* strain GB03 and LB medium, respectively, and treatments with NaCl (0, 100, 200 and 300 mM). The green and white column represent GB03 exposure and control, respectively. Values are means and bars indicate SDs (n = 8). One asterisk (*) and two asterisks (**) indicate $P < 0.05$ and $P < 0.01$ (t test), respectively, for treatment versus controls

levels of the three genes were regulated by GB03 especially in roots under salinity (200 mM NaCl) (Fig. 6a). Compared to corresponding control, GB03 had no effects on the expression of the three genes in shoots except that it significantly down-regulated *PtHKT2*;*1* expression by 59 % ($P < 0.01$) under 200 mM NaCl. Under non-salinity condition, GB03 still had no significant effects on the expression of the three genes in roots, however, it significantly up-regulated *PtHKT1*;*5* and *PtSOS1* expression by 139 % and 83 %, respectively, and down-regulated *PtHKT2*;*1* expression by 42 % ($P < 0.01$) under 200 mM NaCl (Fig. 6b).

Discussions

Growth promotion of *P. tenuiflora* with exposure to commercial *Bacillus* strains

PGPRs have been applied to a wide range of agricultural crops for the purpose of growth enhancement, including improved seed germination and establishment, plant biomass, yields, nutrient uptake efficiency and biotic and abiotic stress tolerance (Zhang et al. 2008a; Harvey et al. 2009; Song and Ryu 2013; de Zelicourta et al. 2013; Gao et al. 2013; Han et al. 2014; Järvan et al. 2014). *B. subtilis* GB03 enhances plant growth, photosynthesis, iron uptake, as well as disease resistance via emission of volatile chemicals (Ryu et al. 2003, 2004; Zhang et al. 2007, 2008a, 2008b, 2009; Zhang et al. 2010). In *Arabidopsis*, GB03 volatile emissions regulated auxin homeostasis and cell expansion (Zhang et al. 2007; Farag et al. 2006; Ryu et al. 2003). It was demonstrated that the introduction of GB03 in the soil triggered wheat biomass accumulation (Zhang et al. 2014). GB03 can enhance the photosynthesis in the leaves of *Arabidopsis thaliana* by reducing photosynthesis of feedback inhibition of glucose and ABA levels (Zhang et al. 2008b). Volatile emissions from GB03 are not merely effective in augmenting short-term growth, photosynthetic capacity and salt tolerance in Petri-dish grown *Arabidopsis* seedlings, but also induce long-term growth promotion, elevated photosynthetic capacity and iron accumulation, as well as delayed albeit

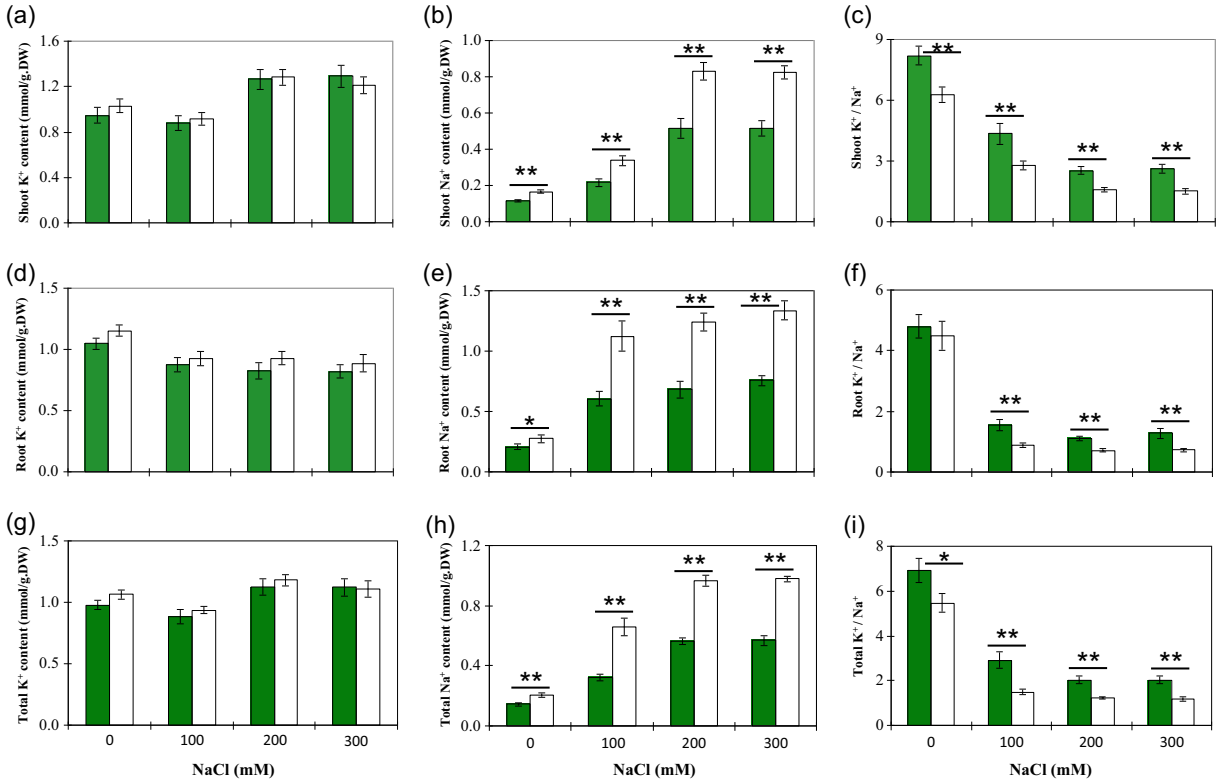

Fig. 4 Effects of *B. subtilis* strain GB03 exposure on Na^+ and K^+ contents and K^+/Na^+ in seedlings of *P. tenuiflora*, **a** shoot K^+ content, **b** shoot Na^+ content, **c** shoot K^+/Na^+, **d** root K^+ contents, **e** root Na^+ content, **f** root K^+/Na^+, **g** Total K^+ contents, **h** total Na^+ level and **i** total K^+/Na^+. Seedling ions were measured and K^+/Na^+ was calculated after four week exposure to *Bacillus subtilis* strain GB03 and LB medium, respectively, and treatments with NaCl (0, 100, 200 and 300 mM). The green and white column represent GB03 exposure and control, respectively. Values are means and bars indicate SDs ($n = 8$). One asterisk (*) and two asterisks (**) indicate $P < 0.05$ and $P < 0.01$ (t test), respectively, for treatment versus controls

higher seed count compared with water-treated control plants (Xie et al. 2009). Soil inoculation with GB03

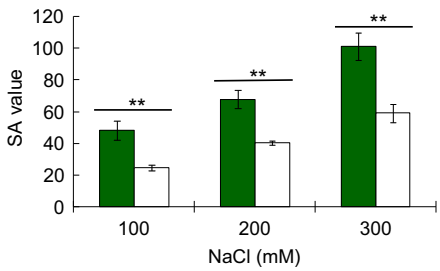

Fig. 5 Effects of *B. subtilis* strain GB03 exposure on selective absorption capacity for K^+ over Na^+ (SA) of *P. tenuiflora*. SA values were calculated according to shoot and root K^+/Na^+ after four week exposure to *Bacillus subtilis* strain GB03 and LB medium, respectively, and treatments with NaCl (100, 200 and 300 mM). The green and white columns represent GB03 exposure and control, respectively. Values are means and bars indicate SDs ($n = 8$). Two asterisks (**) indicates $P < 0.01$ (t test) for treatment versus controls

promotes white clover growth under both non-saline and saline conditions by directly or indirectly regulating plant chlorophyll content, leaf osmotic potential, cell membrane integrity and ion accumulation (Han et al. 2014). Our results indicated that GB03 is more efficient than the other two strains, 2808 and 713, in promoting growth of *P. tenuiflora* roots and shoots; GB03 significantly improved *P. tenuiflora* fresh and dry weights of shoot and root, root length, leaf chlorophyll and leaf chlorophyll a/b.

Salt tolerance of *P. tenuiflora* was enhanced to a higher level by GB03

Plant salt stress due to elevated soil salinity can be quantified by physiological parameters including growth inhibition (e.g. tissue biomass and root length) elevated internal Na^+ and reduced K^+/Na^+ ratios (Zhang et al. 2010). Leaf chlorophyll content is also an

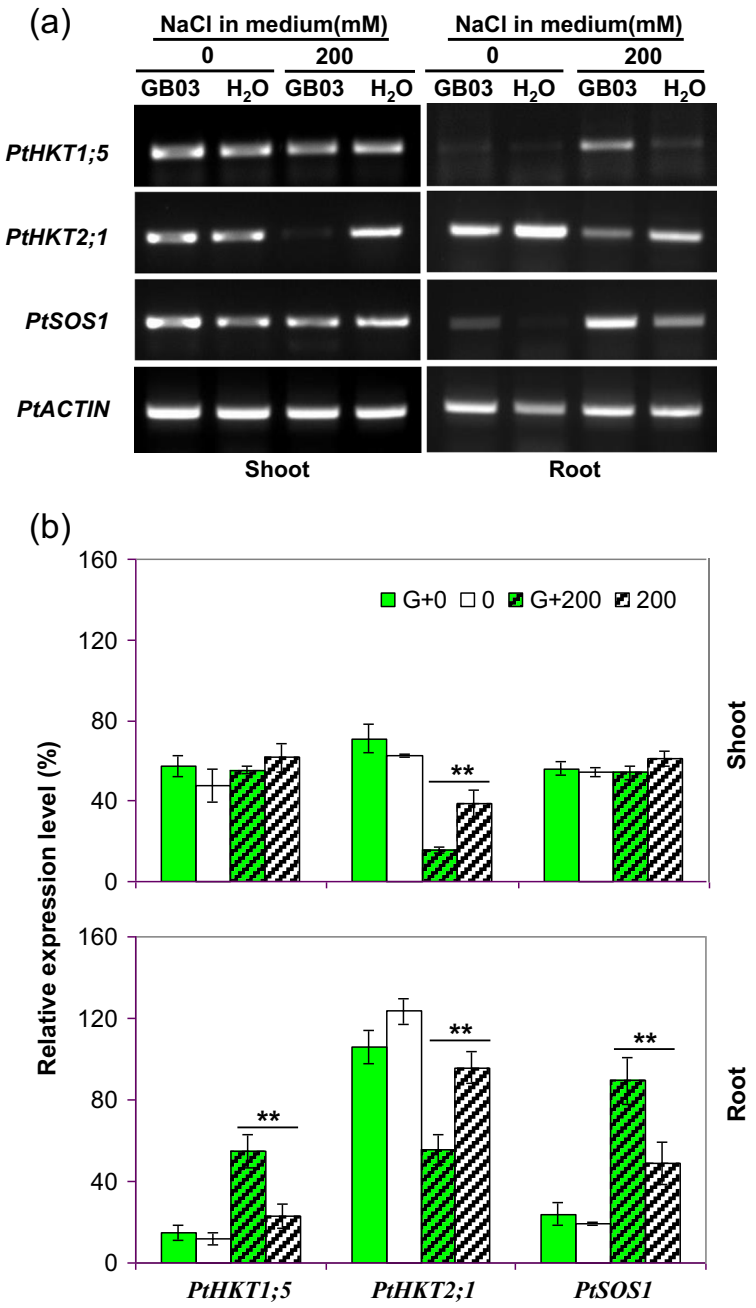

Fig. 6 Tissue-specific expression analysis of *PtHKT1*;*5*, *PtHKT2*;*1* and *PtSOS1* in *P. tenuiflora*. Seedlings were grown in medium with 0 or 200 mM NaCl for two weeks, and then were harvested to extract total RNA after 48 h of exposure to GB03 VOCs before semi-quantitative RT-PCR was performed. (a) RT-PCR gel image assay of *PtHKT1*;*5*, *PtHKT2*;*1* and *PtSOS1* in shoot and root in *P. tenuiflora* under 0 or 200 mM NaCl; (b) Semi-quantitative RT-PCR of *PtHKT1*;*5*, *PtHKT2*;*1* and *PtSOS1*in shoot and root in *P. tenuiflora* under 0 or 200 mM NaCl

important physiological trait directly related to photosynthesis rate in plants (Ma et al. 2012). Previous studies showed that plants under salinity conditions produced less chlorophyll and dry matter than those without salinity stress due to chlorophyll peroxidation (Lunde et al. 2007; Barry 2009). Recently, several reports have started to establish the role of beneficial soil bacteria in improving plant tolerance to abiotic stress, including drought and salinity (Cho et al. 2008; Zhang et al. 2008a; Belimov et al. 2009). It was found that in *Arabidopsis* GB03 decreased whole plant Na^+ content to 54 % of that in control plants (Zhang et al. 2008a). GB03 improved salt tolerance of wheat by decreasing Na^+ accumulation and increasing K^+/Na^+ ratio (Zhang et al. 2014). In the case of white clover, GB03 significantly reduced leaf osmotic potential and improved leaf

chlorophyll content, especially, GB03 decreased shoot and root Na^+ accumulation and thereby improved K^+/Na^+ ratio when GB03-inoculated plants were grown under elevated salt conditions (Han et al. 2014). It was found that *P. tenuiflora* exhibited stronger selective absorption for K^+ over Na^+ than wheat (Wang et al. 2009) and could maintain better K^+ retention and higher K^+ tissue content (Teakle et al. 2013) under saline conditions. With the halophyte species *P. tenuiflora*, NaCl concentrations as high as 300 mM were assayed and GB03 exposure significantly improved plant biomass, decreased whole plant Na^+ content, increased K^+/Na^+ in both shoots and roots with no measurable effect on K^+ content, and therefore, enhanced the selective absorption for K^+ over Na^+.

Contribution of transcriptional regulation of several ion transporters and channel by GB03 to reduced Na^+ transport and uptake from root to shoot

HKT1;5 functions as Na^+ recirculation from shoot to root and HKT2;1 as K^+-Na^+ cotransport (Zhang et al. 2010). In rice, OsHKT1;5 mediates Na^+ removal from the xylem sap into the xylem parenchyma cells (Ren et al. 2005). The association of leaf Na^+ concentrations with the *OsHKT1;5* allele was generally strong, these probably explain the existence of additional highly effective exclusion mechanisms (Platten et al. 2013). In wheat, TaHKT1;5-D, as a Na^+ selective transporter expressed in stellar root cells, can limit the amount of Na^+ transported from xylem to the leaf tissue (Munns et al. 2012). Byrt et al. (2014) reported that TaHKT1;5-D retrieves Na^+ from the xylem vessels in the root and has an critical role in restricting the transport of Na^+ from the root to the leaves and maintaining a high K^+/Na^+ ratio in the leaves in bread wheat. TmHKT1;5-A selectively transports Na^+ and is localized in the plasma membrane of the stellar parenchyma and pericycle cells surrounding xylem vessels where it could easily retrieve Na^+ from the xylem and decrease Na^+ moving to leaves (Munns et al. 2012). Recently, Böhm et al. (2015) found that the presence of serine at S-G-G-G motif in *Dionaea muscipula* HKT1 makes it a highly selective Na^+ channel, not coupled with a movement of any other ions. TaHKT2;1 in wheat mediates Na^+ influx, and its decreased expression leads to an inhibition of this pathway and hence improved ability to cope with saline conditions through decreased tissue Na^+ concentrations (Laurie et al. 2002). OsHKT2;1 is the central transporter for nutritional Na^+ uptake into K^+-starved rice roots thus enhance root growth under K^+-starvation condition (Horie et al. 2007). PtHKT2;1 has a high affinity K^+-Na^+ symport function in yeast and expression pattern analysis of *PtHKT2;1* in *P. tenuiflora* implied that PtHKT2;1 might facilitate K^+ uptake for maintaining a constant K^+/Na^+ ratio under low external K^+ concentration and in the presence of elevated Na^+ (Ardie et al.

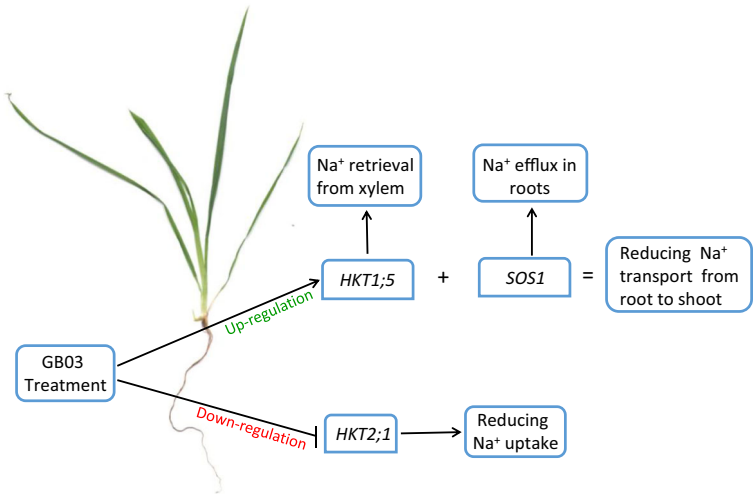

Fig. 7 Model of PtHKT1;5, PtHKT2;1 and PtSOS1-dependant Na^+ transport regulation by GB03. HKT1;5 contributes to Na^+ retrieval from xylem (Ren et al. 2005; Munns et al. 2012; Platten et al. 2013; Byrt et al. 2014) and SOS1 contributes to Na^+ efflux (Wu et al. 1996; Amtmann and Sanders 1999; Qiu et al. 2002; Shi et al. 2002, 2003; Katiyar-Agarwal et al. 2006; Oh et al. 2007; Martinez-Atienza et al. 2007; Zhang et al. 2010; Guo et al. 2012) in roots, and HKT2;1 controls Na^+ uptake in roots (Laurie et al. 2002; Horie et al. 2007; Ardie et al. 2009). Therefore, GB03-triggered up-regulation of *PtHKT1;5* and *PtSOS1* and down-regulation of *PtHKT2;1* in roots reduced Na^+ transport from root to shoot as well as Na^+ uptake in roots

2009). AtHKT1;1 from *Arabidopsis thaliana* has dual functions, Na^+ uptake in roots (Laurie et al. 2002; Rus et al. 2001) and Na^+ recirculation from shoots to roots (Berthomieu et al. 2003; Sunarpi et al. 2005; Davenport et al. 2007). Tissue specific regulation of *AtHKT1*;*1* by GB03 confers salt tolerance in *Arabidopsis thaliana*: under salt stress (100 mM NaCl), GB03 concurrently down- and up-regulates *AtHKT1*;*1* expression in roots and shoots, respectively, resulting less Na^+ entry into plants and enhanced Na^+ retrieval from shoot to root, thereby managing Na^+ equilibrium and conferring salt tolerance (Zhang et al. 2008a). Recently, it was observed that *Bacillus amyloliquefaciens* NBRISN13 confers salt tolerance in rice by modulating differential transcription in a set of at least 14 genes (Nautiyal et al. 2013). In this study, GB03 up-regulated *PtHKT1*;*5* expression, but down regulated *PtHKT2*;*1* expression in roots of *P. tenuiflora* under salt stress (200 mM NaCl).

SOS1 is important for Na^+ efflux at cellular level and controlling long-distance transport of Na^+ from root to shoot in a whole plant (Shi et al. 2000, 2002). Therefore, SOS1 plays a critical role in salt resistance of higher plants: overexpression of *SOS1* in *Arabidopsis* confers increased salt tolerance (Shi et al. 2003). The down-regulation of *Thellungiella halophila ThSOS1* converted the halophyte into a salt-sensitive plant (Oh et al. 2007). transgenic *Arabidopsis* plants overexpressing *SOS1* exhibit increased salt resistance (Yang et al. 2009). It was suggested that SOS1-dependent Na^+ extrusion is an important mechanism to maintain a relatively low Na^+ concentration and high K^+/Na^+ ratio in non-nodulated *Medicago falcata* under saline conditions, thus conferring its salt tolerance (Liu et al. 2015). SOS1 mutation affects intracellular K^+ homeostasis by activating a plasma membrane outward rectifying K^+ channel (Shabala et al. 2005). It was proposed that PtSOS1 is the major component of selective transport capacity for K^+ over Na^+, hence it enhances salt tolerance of *P. tenuiflora* (Guo et al. 2012). Our results indicated that GB03 up-regulated *PtSOS1* expression under salt stress (200 mM NaCl), which is consistent with improved salt tolerance and reduced Na^+ accumulation in shoots, roots and whole plants of *P. tenuiflora*.

Conclusions

Our results presented here established that *B. subtilis* GB03 promoted the growth and improved the salt tolerance of the monocotyledonous halophyte *P. tenuiflora* to a higher level. Interestingly, GB03 regulates several sodium transporter genes (*PtHKT2*;*1*, *PtHKT1*;*5* and *PtSOS1*) that are correlated to improved salt-tolerance. HKT1;5 contributes to Na^+ retrieval from xylem (Ren et al. 2005; Munns et al. 2012; Platten et al. 2013; Byrt et al. 2014) and SOS1 contributes to Na^+ efflux (Wu et al. 1996; Amtmann and Sanders 1999; Qiu et al. 2002; Shi et al. 2002, 2003; Katiyar-Agarwal et al. 2006; Oh et al. 2007; Martinez-Atienza et al. 2007; Zhang et al. 2010) in roots, and HKT2;1 controls Na^+ uptake in roots (Laurie et al. 2002; Horie et al. 2007; Ardie et al. 2009). Therefore, GB03-triggered up-regulation of *PtHKT1*;*5* and *PtSOS1* and down-regulation of *PtHKT2*;*1* in roots reduced Na^+ transport from root to shoot as well as Na^+ uptake in roots (Fig. 7). This study provides the physiological and molecular evidence that application of selected bacteria to salt-tolerant Monocots can ameliorate deleterious effects of high soil saline toxicity.

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