J. Microbiol. Biotechnol. (2016), 26(5), 946–952
http://dx.doi.org/10.4014/jmb.1512.12055

jmb

The Endo-β-1,4-Glucanase of *Bacillus amyloliquefaciens* Is Required for Optimum Endophytic Colonization of Plants

Xiaojing Fan[1], Ruixian Yang[2], Sixin Qiu[3], Xueqing Cai[1], Huasong Zou[1], and Fangping Hu[1]*

[1]*College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, P.R. China*
[2]*Department of Environmental Engineering and Chemistry, Luoyang Institute of Science and Technology, Luoyang, Henan 471023, P.R. China*
[3]*The Crop Institute, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian 350013, P.R. China*

Received: December 21, 2015
Revised: February 5, 2016
Accepted: February 13, 2016

First published online
February 24, 2016

**Corresponding author*
Phone: +86-591-83789469;
Fax: +86-591-83789469;
E-mail: huf@fafu.edu.cn

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

The *eglS* gene in *Bacillus amyloliquefaciens* encodes an endo-β-1,4-glucanase that belongs to glycosyl hydrolase family 5. In this study, a disruption mutant of gene *eglS* was constructed to examine its role in bacterial adaptation in plants. The mutant $TB2_k$, *eglS* gene inactivated bacterial strain, was remarkably impaired in extracellular cellulase activity. When inoculated on *Brassica campestris*, the $TB2_k$ population was reduced by more than 60% compared with the wild-type strain in the root, stem, and leaf tissues. Overexpression of *eglS* in the wild-type strain increased the bacteria population in the plant tissues. Further studies revealed that the transcription level of *eglS* was correlated with bacterial population. These data demonstrate that endo-β-1,4-glucanase of *B. amyloliquefaciens* is required for its optimal endophytic colonization.

Keywords: *Bacillus amyloliquefaciens*, colonization, endophytic, endo-β-1,4-glucanase

Introduction

Endophytic bacteria are defined as bacteria that live intercellularly or intracellularly without causing any apparent damage or disruption to the plant's homeostasis [14]. Endophytic bacteria help promote plant growth by inducing the production of plant hormones, such as auxins, cytokinins, and gibberellins, and providing nutrients, such as nitrogen and phosphorus. They also impart plant resistance to pathogens by inducing plant defense reactions and the biosynthesis of plant antagonistic substances [3, 7, 19, 20]. Based on these mechanisms, endophytic bacteria are proposed as promising biological agents to control plant disease and reduce the usage of agricultural chemicals. The colonization process is pivotal for endophytic bacteria because it determines whether the bacteria can successfully live in the plant.

The endophytic *Bacillus amyloliquefaciens* TB2 is an effective plant growth-promoting bacterial strain isolated from tobacco [13]. In addition to promoting the growth of capsicum, tomato, and other vegetables, the strain TB2 produces active substances that display antimicrobial activity against *Phytophthora capsici*, *Fusarium oxysporum* f.sp. *cucumerinum*, and *Ralstonia solanacearum* [13]. Because *Bacillus* strains are easily processed into stable products, the strain TB2 is a promising biocontrol agent in vegetable production.

Endoglucanase, a members of the glycoside hydrolase family GH5, is thought to be involved in plant colonization by endophytes [4, 12, 15]; however, two independent researchers inferred that no glycoside hydrolases were encoded on the endophytic bacteria *Enterobacter* sp. 638 and *Herbaspirillum seropedicae* SmR1 genomes [11, 17]. In our previous work, the *eglS* gene was characterized from *B. amyloliquefaciens* TB2 and the expression of *eglS* in an *Escherichia coli* strain confirmed that the bacteria have extracellular endo-β-1,4-glucanase activity [5]. To investigate the biological function of endo-β-1,4-glucanase in *B. amyloliquefaciens* TB2, a disruption mutant of gene *eglS* was constructed to test the colonization phenotype in this

Table 1. Strains and plasmids used in this study.

Strain/Plasmid	Description	Source/Reference
Escherichia coli JM109	recA1, endA1, gyrA96, thi-1, hsdR17(rK^-,mK^+), 14^-(*mcrA*$^-$), supE44, relA1, Δ(*lac-proAB*)/F' [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Laboratory stock
B. subtilis 168	*trpC2*	Bacillus Genetic Stock Center
B. amyloliquefaciens		
TB2	Wild type	Laboratory stock
TB2-gfp	*gfp* labeling strain, TB2:: pS4GFP, Km^r	[6]
$TB2_e$	*eglS*-overproducing strain, TB2:: pS401GFP, Km^r	This study
$TB2_k$	*eglS*-deficient strain, TB2:: pMUTIN-$GFP^+_{\Delta eglS}$, Er^r	This study
$TB2_{kc}$	*eglS* gene complementary strain, $TB2_k$:: pS402GFP, Km^r, Er^r	This study
Plasmids		
pGFP-4412	*B. subtilis-E. coli* shuttle vector, Ap^r, Km^r, *gfp*	[18]
pMUTIN-GFP^+	Integration vector, Ap^r, Er^r, *gfp*	[8]
pS401GFP	pGFP-4412 derivative carrying *rpsD* promoter and *eglS*, Ap^r, Km^r, *gfp*	This study
pS402GFP	pGFP-4412 derivative carrying full-length $eglS_f$, Ap^r, Km^r, *gfp*	This study
pMUTIN-$GFP^+_{\Delta eglS}$	pMUTIN-GFP^+ derivative carrying $eglS_p$, Ap^r, Er^r, *gfp*	This study

study. Our findings supported the hypothesis that endo-β-1,4-glucanase EglS was correlated with endophytic colonization by *B. amyloliquefaciens* TB2.

Materials and Methods

Bacterial Strains and Growth Conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was grown at 37°C in Luria-Bertani medium. Strains of *B. amyloliquefaciens* and *B. subtilis* were cultivated in NA broth (0.5% NaCl, 1% bactopeptone, and 0.3% beef broth; pH 7.2) or on 1.8% NA agar at 28°C. When needed, antibiotics were added to the media according to the following concentrations: kanamycin (Km) 20 μg/ml, ampicillin (Ap) 100 μg/ml, and erythromycin (Er) 0.5 μg/ml.

Recombinant DNA Techniques and Mutagenesis

PCR primers were synthesized by Sangon Biotech Co., Ltd.

Table 2. Primers used in this study.

Target		Sequence (5′ to 3′)	Restriction site	Product size (bp)
rpsD	PF	GGAATTCGGCGGATAATTGATCTTTAG	EcoRI	340
	PR	GGAATTCCATATGATGACTCCTCCTTTGG	NdeI	
eglS	F1	GGAATTCCATATGGCAGATATGAAACGGTCAATCTC	NdeI	1,500
	R1	GCTCTAGAGCTTAACTAATTTGGTTCTGTTC	XbaI	
$eglS_p$	Fk	GGGGTACCTATTGATAATCCGTCCGTG	KpnI	215
	Rk	CCATCGATATCACCGTTTGGTTCGT	ClaI	
$eglS_f$	F2	GGAATTCTATAAATATAAAACAAAGCTG	EcoRI	1,802
	R1	GCTCTAGAGCTTAACTAATTTGGTTCTGTTC	XbaI	
	Fyz	AACGCCAGTAGCCAAGA		675
	Ryz	GGATAACGGGAAAAGCA		
16S rRNA	16S-F	GCTCGTGTCGTGAGATGTTGG		198
	16S-R	CGGTTTCGCTGCCCTTTGT		
gfp	G-F	CGTCTGGAAATGGCGGTGTA		128
	G-R	CGGCTTTAACGCTGAGGATG		

Restriction enzyme recognition sites used for vector construction are underlined.

(China) (Table 2). Genomic and plasmid DNAs were isolated by standard protocols [16]. Restriction enzymes, DNA ligase, and other DNA enzymes were used according to the manufacturers' recommendations. Cell preparation and transformation of *B. amyloliquefaciens* TB2 were performed as described by Yasbin *et al*. [22].

To obtain the *eglS* overexpression construct, the constitutive *rpsD* promoter and *eglS* coding regions were amplified by PCR from *B. subtilis* 168 and *B. amyloliquefaciens* TB2 genomic DNA, respectively. Two purified PCR products were ligated after digestion with NdeI, and then the primer pair of PF and R1 (Table 2) was used to amplify a 1,840-bp-long fragment from the ligation mixture. The purified PCR fragments were digested with EcoRI and XbaI, and ligated into plasmid pGFP4412, resulting in plasmid pS401GFP. The construct was transformed into wild-type strain TB2 to create an *eglS* overexpression strain, $TB2_e$. With the aid of the green fluorescent protein gene (*gfp*) in pGFP4412 [18], strain $TB2_e$ could be viewed with a fluorescent microscope with an excitation wavelength of 488 nm.

The *eglS*-inactivated mutant was constructed by homologous recombination using the integration vector pMUTIN-GFP$^+$ [8]. The partial *eglS* gene ($eglS_p$) was amplified with the primer pair of Fk/Rk, and ligated into pMUTIN-GFP$^+$ to generate pMUTIN-GFP$^+_{\Delta eglS}$. The construct pMUTIN-GFP$^+_{\Delta eglS}$ was transformed into wild-type TB2. The desired mutant, $TB2_k$, developed on NA plates containing Er, was confirmed by PCR with Fyz and Ryz primers.

For complementation analysis, primers F2 and R1 were used to amplify a 1,802 bp DNA fragment containing gene *eglS* and its native promoter region. The PCR product, $eglS_f$, was directly cloned into plasmid pGFP4412 at the EcoRI and XbaI sites. The resultant recombinant, pS402GFP, was transformed into a $TB2_k$ strain and generated a complementary strain, $TB2_{kc}$, which was also screened by a fluorescent microscope with an excitation wavelength of 488 nm.

Endo-β-1,4-Glucanase Activity Assays

B. amyloliquefaciens strains were dotted on NA plates containing 0.5% carboxymethyl cellulose (CMC) and 0.01% trypan blue. Endo-β-1,4-glucanase activity was evaluated by the diameter of hydrolysis halo surrounding each colony after incubation at 28ºC for 24 h. Quantitative evaluation was based on the method described by Zhang *et al*. [23]. One unit of endo-β-1,4-glucanase activity was defined as the amount of enzyme that yielded 1 μmol of glucose per minute at 50ºC.

RNA Isolation and qRT-PCR Analysis

Total RNA was extracted from bacterial samples with the SV Total RNA Isolation System (Promega, USA). The first-strand cDNA was synthesized using the PrimeScript One Step RT-PCR Kit (Takara, Japan) according to the manufacturer's instructions. qRT-PCR was performed in triplicates with the SYBR green dye in a MyiQ iCycler thermocycler (Bio-Rad, USA) using the primers listed in Table 2. The calculated Ct was normalized to the Ct of the 16S rDNA transcript from the same cDNA sample before the calculation of fold change using the ΔΔCt method as described by Lathem *et al*. [9]. Statistical analyses were performed using the Student's *t*-test.

Quantification of Endophytic Population in Chinese White Cabbages

To measure the ability of *B. amyloliquefaciens* strains to colonize and proliferate, six germinated Chinese white cabbage seeds were planted in each 24-cm-diameter clean pots, in which the soil was pre-sterilized via autoclaving at 121°C for 30 min to kill the natural microbiomes, and then were cultivated under a climate-controlled greenhouse at 25°C, relative humidity of 80%, and a cycle of 12 h light and 12 h darkness. Then 30-day-old Chinese white cabbage seedlings were inoculated with the bacteria liquid (10^8 CFU/ml) using the irrigation method. Sterile distilled water was used as a control. Bacterial populations were quantified from the plant roots, stems, and leaves at 2, 5, 10, 20, and 30 days after inoculation. Six plants were sampled for bacterial colonization per treatment, and a total of 450 plants were used in three replicates.

The method for isolation of endophytic bacteria from plant tissues was performed according to Yang *et al*. [21]. The final plant macerates were serially diluted and 100 μl was spread onto NA solid media containing the corresponding antibiotics. The colony forming units (CFU) were counted in 48 h after incubation at 28°C.

Results

Construction of *B. amyloliquefaciens* Mutant Strains with Selection Marker

To examine the *eglS* disruption mutant and ascertain the integrated position on the chromosome of TB2, primers Fyz and Ryz were designed. Since the integrated vector pMUTIN-GFP$^+_{\Delta eglS}$ was expected to insert into the chromosome at the *eglS* site via homologous single crossover, the forward primer Fyz was designed on the chromosome of TB2 and the reverse primer Ryz was anchored on the *gfp* gene of the pMUTIN-GFP$^+$ plasmid. As expected, a 675 bp DNA fragment was amplified from mutant DNA (Fig. 1); however, no product was obtained when wild-type TB2 or plasmid pMUTIN-GFP$^+$ was used as templates (Fig. 1). Sequence analysis of the PCR product derived from mutant $TB2_k$ confirmed that pMUTIN-GFP$^+_{\Delta eglS}$ was inserted into the 519 bp downstream of the translation start site (ATG) of *eglS* protein by single-crossover.

Colonies of the overexpression strain $TB2_e$ and complementary strain $TB2_{kc}$ emitted strong fluorescence under a fluorescent microscope on the NA plates containing Km (Fig. 2). The stability of plasmids in $TB2_e$ and $TB2_{kc}$ was also evaluated. After 30 generations of continuous culturing in antibiotic-free NA broth, 96% of the transformed cells of

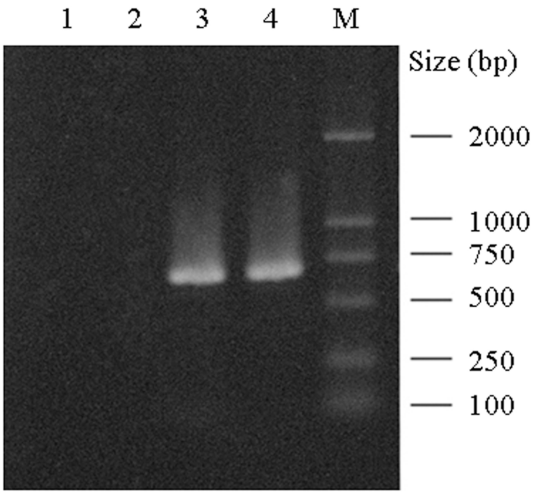

Fig. 1. The deficient mutant $TB2_k$ was identified by PCR using primers Fyz and Ryz.
Lane M: DL 2000 Plus DNA Marker; Lane 1: PCR product amplified with plasmid pMUTIN-GFP$^+$ as the template; Lane 2: PCR product of TB2 as the template; Lanes 3 and 4: PCR product of $TB2_k$ as the template with a band at 675 bp.

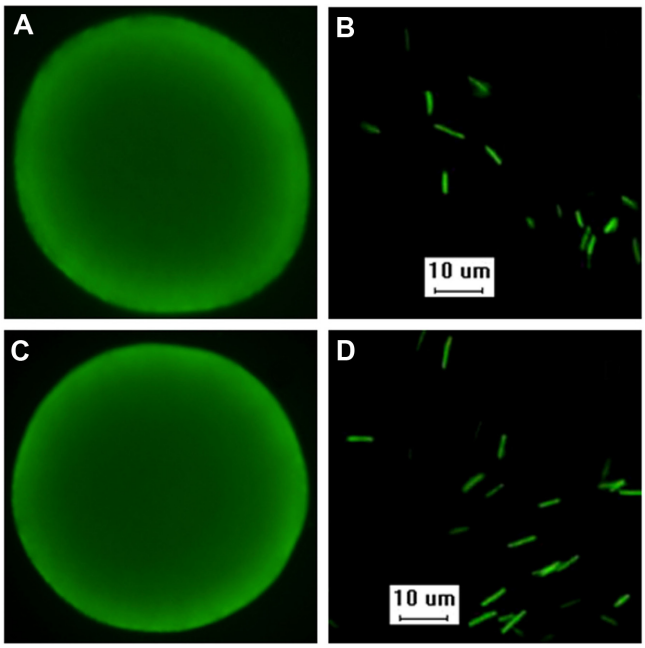

Fig. 2. Green fluorescence colonies and cells of $TB2_e$ and $TB2_{kc}$ upon excitation at 488 nm under a fluorescent microscope.
(**A**) Bacterial colony of $TB2_e$; (**B**) Cells of $TB2_e$; (**C**) Bacterial colony of $TB2_{kc}$; (**D**) Cells of $TB2_{kc}$.

$TB2_e$ and $TB2_{kc}$ retained kanamycin resistance (Fig. S1), which suggested that plasmids pS401GFP and pS402GFP were stable in the tested *B. amyloliquefaciens* strains.

Mutant $TB2_k$ Shows Weak Endo-β-1,4-Glucanase Activity

In order to test the endo-β-1,4-glucanase activity, *B. amyloliquefaciens* strain TB2-gfp was used as a control in activity assays, which was a *gfp*-labeled strain in our laboratory [6]. The strain $TB2_e$ that overexpressed *eglS* showed a large and dual hydrolytic circle compared with the control strain TB2-gfp. The deficient mutant $TB2_k$ showed a smaller and very weak hydrolytic circle, which indicated that endo-β-1,4-glucanase activity was significantly reduced. The endoglucanase activity was restored in the complementary strain $TB2_{kc}$, whose hydrolytic circle was

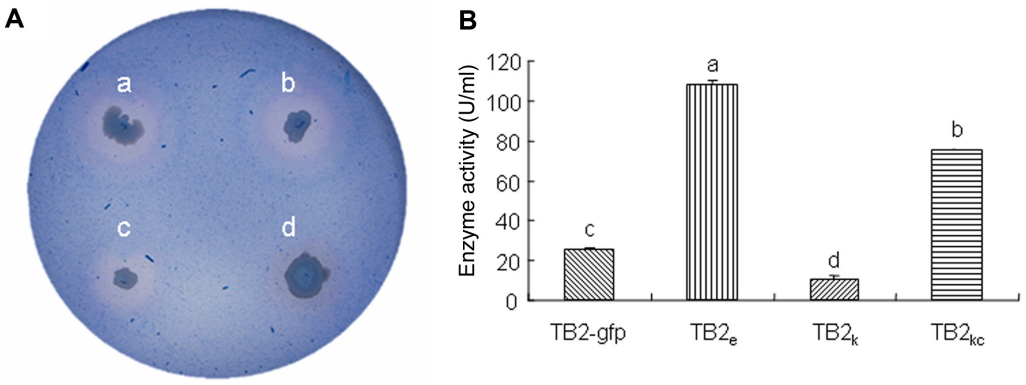

Fig. 3. Endo-β-1,4-glucanase activity assays.
(**A**) Qualitative detection of endo-β-1,4-glucanase activity in different strains on CMC agar plate. a, $TB2_{kc}$; b, $TB2_e$; c, TB2-gfp; d, $TB2_k$. (**B**) Quantitative analysis of endo-β-1,4-glucanase activity. Student's *t*-test ($p < 0.05$) was used to analyze the data. Error bars above columns give standard deviations from at least three transformants per treatment. The lowercase letters indicate values, with "a" being the highest and "d" the lowest value.

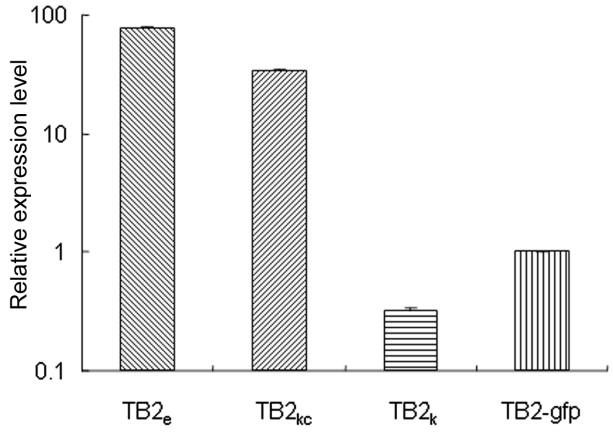

Fig. 4. Quantitative RT-PCR analysis of endo-β-1,4-glucanase gene expression in $TB2_e$, $TB2_{kc}$, $TB2_k$, and TB2-gfp.

larger than that of TB2-gfp, and showed almost the same phenotype as overexpressed strain $TB2_e$ (Fig. 3A).

In addition to qualitative detection, quantitative assays demonstrated that the $TB2_e$ strain displayed the greatest endo-β-1,4-glucanase activity, producing endoglucanase 4.2-fold higher than that of TB2-gfp (Fig. 3B). The complementary strain $TB2_{kc}$ activity was 3.6-fold higher than TB2-gfp, whereas the endo-β-1,4-glucanase activity of mutant $TB2_k$ was significantly lower than that of the other three strains ($p < 0.05$).

Transcriptional Expression of *eglS* in *B. amyloliquefaciens* Strains

Following normalization of the expression levels of *eglS* to constitutively expressed 16S rDNA, we observed a high-level transcription of *eglS* in $TB2_e$, which was carrying multicopy *eglS*. The relative mRNA level in the $TB2_e$ strain was 77-fold higher than that in the control strain TB2-gfp (Fig. 4). The mRNA level in mutant $TB2_k$ was remarkably reduced at only 0.15-fold of that in TB2-gfp. In complemented strain $TB2_{kc}$, the mRNA level of *eglS* was 35-fold higher than that in TB2-gfp.

eglS Affects the Colonization Ability of *B. amyloliquefaciens* in Chinese White Cabbage

Examination of the colonization ability of the TB2-gfp, $TB2_e$, $TB2_k$, and $TB2_{kc}$ strains in plants showed that all these strains could be isolated from the plant tissues 2 days after inoculation (Fig. 5). Among the four strains, the population of strain $TB2_e$ was the highest in all plant tissues at each testing time point, where the highest level was reached on the 5th day, and the population was at a level of

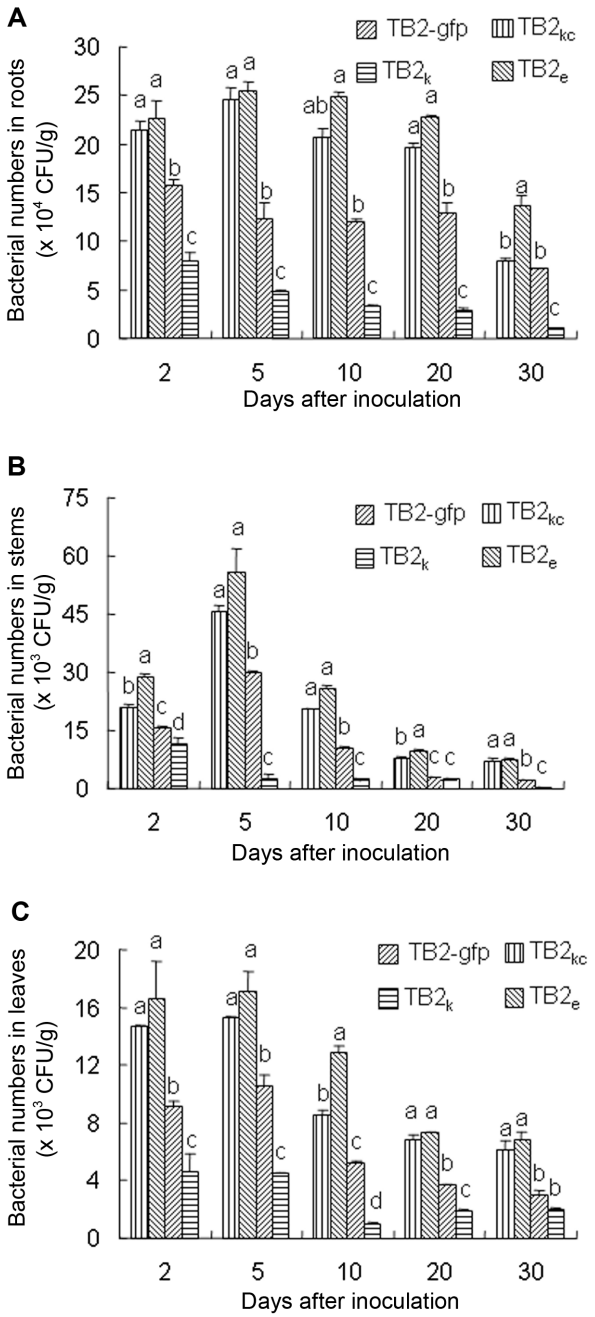

Fig. 5. Population of TB2 modification strains in the roots, stems, and leaves of Chinese white cabbage.
(**A**) Root tissue; (**B**) Stem tissue; **C**, Leaf tissue. The columns represent mean numbers of CFU isolated from the plant, and the bars represent the standard errors of the means resulting from three replicate treatments. Columns that are significantly different from each other ($p < 0.05$) in one experiment are headed by different letters.

2.5×10^5 CFU/g (fresh weight) in the root tissue (Fig. 5A), 5.6×10^4 CFU/g (fresh weight) in the stem tissue (Fig. 5B),

and 1.7×10^4 CFU/g (fresh weight) in the leaf tissue (Fig. 5C). By contrast, the bacterial numbers of mutant $TB2_k$ were significantly lower than those of TB2-gfp, $TB2_e$, and $TB2_{kc}$ ($p < 0.05$), and on the 5th day, the population was 4.7×10^4 CFU/g (fresh weight) in the root tissue (Fig. 5A), 2.8×10^3 CFU/g (fresh weight) in the stem tissue (Fig. 5B), and 4.5×10^3 CFU/g (fresh weight) in the leaf tissue (Fig. 5C). The bacteria populations of the complementary strain $TB2_{kc}$ were restored and even higher than the TB2-gfp. No bacteria could be grown on the NA plates containing Er or Km from the control plants throughout the experiments.

The growth proficiency of $TB2_e$, $TB2_k$, and $TB2_{kc}$ mutants were compared with strain TB2-gfp in NA broth (Fig. S2). Although the entry into the stationary phase of the three mutants almost lagged behind the TB2-gfp strain by 2 h, the generation times of the four strains were similar (*i.e.*, 1.0 h). The result suggests that the colonization ability change is not due to growth proficiency, but altered by the endo-β-1,4-glucanase EglS.

Discussion

Endoglucanase might be involved in endophytic colonization of *B. amyloliquefaciens* in plants. To examine the role of endoglucanase in colonization, a disruption strain and an overexpression strain were constructed and inoculated into *Brassica campestris* plants. The bacteria populations collected from plant tissues were closely related with the endo-β-1,4-glucanase activity of the bacteria. Mutation in the endo-β-1,4-glucanase gene (*eglS*) affected bacteria colonization in Chinese white cabbage. Bacteria populations of the defective strain collected from cabbage root, stem, and leaf tissues were remarkably smaller compared with the control strain, which was consistent with previous works on endophytic bacteria [15]. *B. amyloliquefaciens* did not cause any apparent damage to plant growth despite the high endo-β-1,4-glucanase activity of $TB2_e$ and $TB2_{kc}$ (data not shown).

In Chinese white cabbage root, stem, and leaf tissues, the population density increased between the 2nd and 5th days after inoculation, and then decreased from the 10th to the 30th day, and the highest population density occurred at the 5th day after inoculation. This suggested a balanced ecosystem in the interior plant tissue, which had a suppression effect on *B. amyloliquefaciens* growth. A possible explanation may be that endophytes generally trigger low levels of defense responses to prevent certain damage on plant homeostasis, which may restrict the endophyte's capability to multiply in plants [14]. The biological function of endoglucanase in endophytes is mainly for sugar utilization, which is quite different from plant pathogenic bacteria [10].

Although endo-β-1,4-glucanase is involved in plant colonization, it is not required for all endophytes. Previous researchers have reported the penetration roles of endoglucanase in *Burkholderia* sp. and *Azoarcus* sp. BH72 [4, 15]. In grapevine (*Vitis vinifera*), the polygalacturonidase and endoglucanase of *Xylella fastidiosa* enlarged the pore sizes of pit membranes, which presumably facilitated the systemic spread of bacteria in the plants [12]. However, colonization and penetration in *H. seropedicae* SmR1 appear to involve bacterial lipopolysaccharide [1], the type three secretion system, and exopolysaccharide [10], but not necessarily the involvement of cell wall-degrading enzymes, because in silico analysis of the *H. seropedicae* SmR1 genome did not reveal genes coding for known cellulases, pectinases, or any other cell wall-degrading enzymes [11]. Likewise, the endophytic bacteria *Enterobacter* sp. 638 isolated from poplar does not possess any glycoside hydrolases commonly used to break down plant cell wall polymers [17].

It was clear that there was more than one cellulase in *B. amyloliquefaciens* because hydrolytic activity tests on CMC plates or liquid suspension indicated that cellulase activity was not completely impaired in *eglS* disruption mutant $TB2_k$. A number of endophytic bacteria possess multiple copies of cellulase genes. For instance, Cho *et al.* [2] purified two cellulases, Cel5A and Cel5B, from the endophytic bacterial strain *Paenibacillus polymyxa* GS01. Further studies are needed to identify other cellulases in *B. amyloliquefaciens*.

In conclusion, our present study demonstrated that endo-β-1,4-glucanase EglS in endophytic *B. amyloliquefaciens* TB2 was responsible for effective colonization in plants, and mutation of *eglS* resulted in a great reduction of population density in Chinese white cabbage.

Acknowledgments

We are very grateful to Dr. Daniel R. Zeigler (The Ohio State University Department of Microbiology Columbus, OH, United States) for kindly providing the plasmid pMUTIN-GFP$^+$. This work was supported by the National Natural Science Foundation of China (30671463).

References

1. Balsanelli E, Serrato RV, de Baura VA, Sassaki G, Yates MG,

Rigo LU, *et al*. 2010. *Herbaspirillum seropedicae rfbB* and *rfbC* genes are required for maize colonization. *Environ. Microbiol*. **12:** 2233-2244.
2. Cho KM, Hong SJ, Math RK, Islam SM, Kim JO, Lee YH, *et al*. 2008. Cloning of two cellulase genes from endophytic *Paenibacillus polymyxa* GS01 and comparison with *cel 44C-man* 26A. *J. Basic Microb*. **48:** 464-472.
3. Compant S, Clément C, Sessitsch A. 2010. Plant growth-promoting bacteria in the rhizo and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem*. **42:** 669-678.
4. Compant S, Reiter B, Sessitsch A, Nowak J, Clément C, Ait Barka E. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Appl. Environ. Microbiol*. **71:** 1685-1693.
5. Fan XJ, Qiu SX, Hu FP. 2008. Cloning and expression of β-1,4-endoglucanase gene from endophytic *Bacillus amyloliquefaciens* TB2. *Chin. J. Trop. Crops* **29:** 443-449.
6. Fan XJ, Qiu SX, Wu XP, Hong YC, Cai XQ, Hu FP. 2007. Endophytic *Bacillus subtilis* strain BS-2 labeled with green fluorescent protein gene. *Chin. J. Appl. Environ. Biol*. **13:** 530-534.
7. Iniguez AL, Dong Y, Carter HD, Ahmer BM, Stone JM, Triplett EW. 2005. Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant Microbe Interact*. **18:** 169-178.
8. Kaltwasser M, Wiegert T, Schumann W. 2002. Construction and application of epitope- and green fluorescent protein-tagging integration vectors for *Bacillus subtilis*. *Appl. Environ. Microbiol*. **68:** 2624-2628.
9. Lathem WW, Price PA, Miller VL, Goldman WE. 2007. A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science* **315:** 509-513.
10. Monteiro RA, Balsanelli E, Wassem R, Marin AM, Brusamarello-Santos LCC, Schmidt MA, *et al*. 2012. *Herbaspirillum*-plant interactions: microscopical, histological and molecular aspects. *Plant Soil* **356:** 175-196.
11. Pedrosa FO, Monteiro RA, Wassem R, Cruz LM, Ayub RA, Colauto NB, *et al*. 2011. Genome of *Herbaspirillum seropedicae* strain SmR1, a specialized diazotrophic endophyte of tropical grasses. *PLoS Genet*. **5:** e1002064.
12. Pérez-Donoso AG, Sun Q, Roper MC, Greve LC, Kirkpatrick B, Labavitch JM. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa* infected grapevines. *Plant Physiol*. **152:** 1748-1759.
13. Qiu SX, He H, Ruan HC, Guan X, Hu FP. 2004. Selection of endophytic bacteria with antipathogenic and plant growth promoting functions. *Chin. J. Appl. Environ. Biol*. **10:** 655-659.
14. Reinhold-Hurek B, Hurek T. 2011. Living inside plants: bacterial endophytes. *Curr. Opin. Plant Biol*. **14:** 435-443.
15. Reinhold-Hurek B, Maes T, Gemmer S, Van Montagu M, Hurek T. 2006. An endoglucanase is involved in infection of rice roots by the not-cellulose-metabolizing endophyte *Azoarcus* sp. strain BH72. *Mol. Plant Microbe Interact*. **19:** 181-188.
16. Sambrook J, Russell DW. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
17. Taghavi S, van der Lelie D, Hoffman A, Zhang YB, Walla MD, Vangronsveld J, *et al*. 2010. Genome sequence of the plant growth promoting endophytic bacterium *Enterobacter* sp. 638. *PLoS Genet*. **6:** e1000943.
18. Tian T, Qi XC, Wang Q, Mei RH. 2004. Colonization study of GFP-tagged *Bacillus* strains on wheat surface. *Acta Phytopathol. Sin*. **34:** 346-351.
19. Vendan RT, Yu YJ, Lee SH, Rhee YH. 2010. Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. *J. Microbiol*. **48:** 559-565.
20. Weyens N, van der Lelie D, Taghavi S, Vangronsveld J. 2009. Phytoremediation: plant-endophyte partnerships take the challenge. *Curr. Opin. Biotechnol*. **20:** 248-254.
21. Yang RX, Fan XJ, Cai XQ, Hu FP. 2015. The inhibitory mechanisms by mixtures of two endophytic bacterial strains isolated from *Ginkgo biloba* against pepper phytophthora blight. *Biol. Control* **85:** 59-67.
22. Yasbin RE, Wilson GA, Young FE. 1975. Transformation and transfection in lysogenic strains of *Bacillus subtilis*: evidence for selective induction of prophage in competent cells. *J. Bacteriol*. **121:** 296-304.
23. Zhang Y-HP, Hong J, Ye X. 2009. Cellulase assays. *Methods Mol. Biol*. **581:** 213-231.