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

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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.

Endogluconses, a members of the glycosyl hydrolase family 5, is thought to be involved in plant colonization [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...
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.
(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 Ndel, and then the primer pair of PF and RI (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, TB2g. With the aid of the green fluorescent protein gene (gfp) in pGFP4412 [18], strain TB2g 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), was amplified with the primer pair of Fk/Rk, and ligated into pMUTIN-GFP to generate pMUTIN-GFPeglS. The construct pMUTIN-GFPeglS was transformed into wild-type TB2. The desired mutant, TB2ΔeglS, 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, eglSΔ, was directly cloned into plasmid pGFP4412 at the EcoRI and XbaI sites. The resultant recombinant, pS402GFP, was transformed into a TB2Δ strain and generated a complementary strain, TB2ΔeglS, 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 28°C 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 50°C.

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 Latham 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 pot, 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 (10⁶ 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-GFPeglS 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 confirmed that pMUTIN-GFPeglS was inserted into the 519 bp downstream of the translation start site (ATG) of eglS protein by single-crossover.

Colonies of the overexpression strain TB2g and complementary strain TB2ΔegaT emitted strong fluorescence under a fluorescent microscope on the NA plates containing Km (Fig. 2). The stability of plasmids in TB2g and TB2ΔegaT was also evaluated. After 30 generations of continuous cultivating in antibiotic-free NA broth, 96% of the transformed cells of
TB2e and TB2ke retained kanamycin resistance (Fig. S1), which suggested that plasmids pS401GFP and pS402GFP were stable in the tested *B. amyloliquefaciens* strains.

**Mutant TB2e 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 TB2e that overexpressed *eglS* showed a large and dual hydrolytic circle compared with the control strain TB2-gfp. The deficient mutant TB2k 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 TB2kc, whose hydrolytic circle was

![Fig. 1](image1.png)

**Fig. 1.** The deficient mutant TB2k 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 TB2e as the template with a band at 675 bp.

![Fig. 2](image2.png)

**Fig. 2.** Green fluorescence colonies and cells of TB2e and TB2ke upon excitation at 488 nm under a fluorescent microscope. (A) Bacterial colony of TB2e; (B) Cells of TB2e; (C) Bacterial colony of TB2ke; (D) Cells of TB2ke.

![Fig. 3](image3.png)

**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, TB2ke; b, TB2e; c, TB2-gfp; d, TB2k. (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.
larger than that of TB2-gfp, and showed almost the same phenotype as overexpressed strain TB2e (Fig. 3A).

In addition to qualitative detection, quantitative assays demonstrated that the TB2e 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 TB2kc activity was 3.6-fold higher than TB2-gfp, whereas the endo-β-1,4-glucanase activity of mutant TB2k 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 TB2e, which was carrying multicopy eglS. The relative mRNA level in the TB2e strain was 77-fold higher than that in the control strain TB2-gfp (Fig. 4). The mRNA level in mutant TB2k was remarkably reduced at only 0.15-fold of that in TB2-gfp. In complemented strain TB2kc, 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, TB2e, TB2k, and TB2kc 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 TB2e 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 \(2.5 \times 10^5\) CFU/g (fresh weight) in the root tissue (Fig. 5A), \(5.6 \times 10^4\) CFU/g (fresh weight) in the stem tissue (Fig. 5B),
and $1.7 \times 10^4$ 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_k ($p < 0.05$), and on the 5th day, the population was $4.7 \times 10^3$ CFU/g (fresh weight) in the root tissue (Fig. 5A), $2.8 \times 10^3$ CFU/g (fresh weight) in the stem tissue (Fig. 5B), and $4.5 \times 10^3$ CFU/g (fresh weight) in the leaf tissue (Fig. 5C). The bacteria populations of the complementary strain TB2_e 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_k 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, and TB2_k (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* involved to 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 *Xylella fastidiosa* 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. 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).

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