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RESEARCH ARTICLE

Plant growth-promoting activity of beta-propeller protein YxaL secreted from *Bacillus velezensis* strain GH1-13

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Abstract

YxaL is conserved within the *Bacillus subtilis* species complex associated with plants and soil. The mature YxaL protein contains a repeated beta-propeller domain, but the subcellular location and function of YxaL has not been determined. The gene encoding the mature YxaL protein was PCR amplified from genomic DNA of *B. velezensis* strain GH1-13 and used for recombinant protein production. A rabbit polyclonal antibody against the purified YxaL was generated and used for western blotting to determine the constitutive expression and secretion of YxaL. During normal culture growth of strain GH1-13, levels of the constitutively secreted YxaL were slowly rising to 100 μg L^{-1}, and degraded with a half-life of 1.6 h in the culture medium. When the effects of YxaL on plant seed germination and seedling growth were examined, it was shown that seed treatment of *Arabidopsis thaliana* and rice (*Oryza sativa* L.) with purified YxaL at the optimal concentration of 1 mg L^{-1} was effective at improving the root growth of plants. Seedlings from the treated *Arabidopsis* seeds markedly increased transcription of a 1-aminocyclopropane-1-carboxylate synthetase marker gene (ACS11) but reduced expression of auxin- and abscisic acid-responsive marker genes (IAA1, GH3.3, and ABF4), especially when provided with exogenous auxin. Horticulture experiments showed that pepper (*Capsicum annuum*) seeds treated with 1 mg L^{-1} YxaL in a soaking solution increased shoot growth and improved tolerance to drought stress. We hypothesize that YxaL secreted from plant growth-promoting *Bacillus* cells has a significant impact on plant roots, with the potential to improve plant growth and stress tolerance.

Introduction

The protein YxaL has been observed to interact with the DNA helicase PcrA in *Bacillus subtilis* [1]. This interaction can enhance the effectiveness of PcrA *in vitro*, but it is difficult to observe because the amino acid sequence of YxaL (formerly named YxaK) contains a signal peptide (1–44 amino acid residues) at the *N*-terminus for translocation across the cytoplasmic

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membrane [2]. Thus, it is difficult to deduce the site of subcellular interaction between the extracellular protein YxaL and the intracellular helicase PcrA (or DNA). After removing the *N*-terminal signal peptide, the mature YxaL protein (45–415 a.a.) is predicted to contain a repeated pyrrolo-quinoline quinone (PQQ) domain that forms a beta-propeller structure [1]. Beta-propeller proteins have diverse functions with different blade numbers [3]. Based on the sequence homology and structural similarity, it is postulated that beta-propeller homologs with different structural architectures of the blades may have originated from one ancestral blade, most likely that of a PQQ motif beta-propeller [4]. This beta-propeller domain is ubiquitous in diverse proteins with a similar beta-propeller fold observed in methanol dehydrogenase, which uses PQQ as cofactor [5]. In methanol dehydrogenases of methylotrophic bacteria, adjacent cysteine residues in active sites can form disulphide bridges for electron transfer reactions [6, 7]. In contrast, YxaL homologs in *Bacillus* species have no conserved cysteine residue, which suggest that their functions differ from that of PQQ-containing enzymes.

B. velezensis strain GH1-13 was isolated from rice paddy soil in Korea and can promote plant growth and suppress several pathogens [8]. This strain was shown to produce indole 3-acetic acid (IAA) associated with promoted growth of rice root [9]. The chromosome sequence of strain GH1-13 (GenBank accession number CP019040.1) contains highly homologous genes responsible for the biosynthesis of some plant hormones and secondary metabolites, such as IAA, 2,3-butanediol, non-ribosomal lipopeptides, and polyketide antimicrobials, which are believed to be more proficient for rhizosphere colonisation and pathogen control than other members of the *B. subtilis* group [10–12]. In this paper, comparative gene analysis showed that *B. velezensis* strain GH1-13 contains a highly conserved sequence of the *yxaL* gene in the operational group of *B. amyloliquefaciens-velezensis-siamensis* within the *B. subtilis* species complex [13]. This gene is located in an operon composed of two genes, which were designated *yxaJL* in a previous study [14], but its expression pattern and function has not been defined.

The goal of this study was to investigate the expression, localization and effect of YxaL on plant growth. The gene coding for the mature portion of YxaL (45–415 a.a.) was amplified from the genomic DNA of strain GH1-13 by PCR, and then cloned and transformed into *Escherichia coli* to overproduce the protein. The purified protein was utilized as antigen to generate a rabbit polyclonal antibody for detection of YxaL by western blotting and applied for seed treatment of *Arabidopsis thaliana*, rice, and pepper in order to evaluate the effect on plant growth. We report that YxaL was secreted from the *Bacillus* cells into the medium and had a positive effect on the growth of plant roots in the sub-nanomolar range.

Materials and methods

Strains and cultivation

B. velezensis strain GH1-13 was revived from frozen stocks in 50% glycerol at -80˚C and streaked onto tryptic soy agar (BD, Sparks, USA) plates. Single colonies were cultivated in tryptic soy broth (TSB) at 25˚C with aeration (180 rpm), and bacterial growth was assessed by measuring the optical density at 600 nm. For plant culture experiments, *A. thaliana*, rice (*Oryza sativa* L.), and pepper (*Capsicum annuum*) seeds were disinfected with 2% hypochlorite and 0.05% Triton-X for 10 min at room temperature (25˚C). Subsequently, the seeds were washed several times with sterile water, and then treated with various concentrations of purified YxaL (0 to 100 mg L^{-1}) in a soaking solution for 2 h at room temperature. Treated seeds of *A. thaliana* and rice were planted on 0.5% NuSieve GTG agarose (FMC Bioproduct, Rockland, USA) plates containing 1% sucrose, and 0.05% MES buffer (pH 5.7) in 0.5× Murashige & Skoog (MS) medium [15], which was supplemented with or without indole-3-acetic acid

(Sigma, St Louis, USA) at the final concentration of 0.5 μM. Pepper seeds were treated in the soaking solution with or without 1 mg L^{-1} YxaL for 2 h in room temperature, and dried for 30 min under an air stream in a clean bench, and then were planted in each 5 replicates of plant tray (50 cases) containing horticultural soil with a 1:1 mixture of Baroker (Seoul Bio, Eumseong, Korea) and BM6 (Berger, Saint-Modeste QC, Canada).

Cloning and expression of the *yxaL* gene

Genomic DNA of strain GH1-13 was extracted using a Wizard Genomic DNA Purification kit (Promega, Madison, USA). A DNA fragment coding for the mature YxaL protein (45–415 a. a.) was amplified by PCR with the following primers: forward 5′-GGCCCATGGCGGAAACGG TATTTAAACAAAAT and reverse 5′-GGGCTCGAGTTATTTTTTTGCCCCGAATGCGA. The underlined *Nco*I and *Xho*I restriction sites are compatible with those in the plasmid pProEX-HTA (Invitrogen, Eugene, USA) with an *N*-His tag linked to a TEV protease cleavage site. After cloning the *yxaL* gene, the resulting plasmid pProEX-YxaL (*N*-His-TEV) was transformed into *E. coli* strain BL21 (DE3). The transformed cells were grown to an optical density of ~0.5 in 1 L of LB broth containing 100 mg ampicillin with aeration (180 rpm) at 37˚C, after which the recombinant YxaL protein was overexpressed due to the addition of 0.2 mM IPTG for 1 h. Cells were harvested, treated with 1 mM mercaptoethanol and a protease cocktail (Roche Diagnostics, Indianapolis, USA), and disrupted by repeated ultrasonication in an ice-water bath. Unless otherwise stated, protein purification was performed at 4˚C. After centrifugation at 21,000 ×*g* for 15 min, the supernatant was transferred to a new vessel, mixed with 20 mM imidazole and Ni NTA agarose (Qiagen, Hilden, Germany), and agitated on a rotary shaker for 1 h. The protein-bound agarose was loaded on a column, washed with 25 mM imidazole in 1×PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1000 mL water, pH 7.4), and the majority of the YxaL (*N*-His-TEV) was eluted within the fractions containing 100 to 150 mM imidazole (S2A Fig). After exchanging the buffer with 1×PBS using a G25 Sepharose desalting column, the purified protein was mixed with a His-tagged TEV protease at a ratio of 100:1 to remove the *N*-His-TEV site of the recombinant protein, as described previously [16]. The majority of the protein with the removed His tag was applied to an Ni NTA column and was eluted in the unbound fraction. The purified protein size and purity was determined by size exclusion chromatography and SDS PAGE.

Western blotting

Purified YxaL was utilized as antigen to generate a rabbit polyclonal antibody for detecting YxaL by western blotting in subcellular compartments of strain GH1-13 during its growth in TSB medium. A purified anti-YxaL IgG was used as a primary antibody (dilution rate, 1:20,000), which was then detected using a secondary chicken anti-rabbit IgG antibody with horseradish peroxidase (HRP) conjugate (Abcam, Cambridge, UK) and a western blotting detection kit (Advansta, Menlo Park, USA). Chemiluminescent images were acquired using a ChemiDoc XRS image analyser (Bio-Rad, Hercules, USA), and images were manipulated using Molecular Dynamics ImageQuant version 5.2 (GE Healthcare, Waukesha, USA).

Gene expression analysis

To determine the expression levels of the *yxaL* gene during different growth phases of *B. velezensis* strain GH1-13 (GenBank accession number CP019040.1, chromosome) and the phytohormone-responsive genes (IAA1, GH3.3, ACS11, and ABF4; ref. [17]) in 1-week-old cultured roots of YxaL-treated *Arabidopsis* seeds, RNA was extracted using Qiagen RNeasy mini kits. The cDNA was synthesized using a Qiagen QuantiTect Reverse Transcription Kit and

quantitative PCR (qPCR) was performed using a Qiagen QuantiTect SYBR Green PCR Kit with a Roche LightCycler Nano instrument. Relative expression levels of the *B. velezensis* gene (*yxaL*) normalized to reference 16S rRNA levels [18] and the *A. thaliana* genes (IAA1, GH3.3, ACS11, and ABF4) normalized to reference 18S rRNA levels [19] were calculated by the ΔΔCq method [20]. The qPCR primers used are presented in S1 Table.

Plant growth testing

To test effects of YxaL on plant growth, seeds of pepper (*C. annuum*) treated with or without 1 mg L^{-1} YxaL were cultivated in a greenhouse under normal watering conditions for 54 days to measure the length and biomass of shoots and roots. In order to examine the effect of YxaL on drought stress tolerance, the seedlings grown for 58 days in potted soil were deprived of water for 5 days, then re-watered, at intervals of 7 days and monitored for growth of leaves.

Statistics

Experimental data from at least three independent replicates were reported as the means and standard deviation of the means. Significant differences between data were determined by analysis of variance (ANOVA) and *t*-tests, with differences considered to be significant at a *P*-value of less than 0.05.

Results

Evolutionary relationships of YxaL with beta-propeller domains in bacteria

A database search for YxaL homologs in bacteria showed that the amino acid sequence of YxaL obtained from *B. velezensis* strain GH1-13 is highly conserved within *Bacillus* species. The YxaL homologs have diverged into two types, tentatively named YxaL1 and YxaL2, which are present in distinct operational taxonomic groups of *B. amyloliquefaciens-siamensis-velezensis* and *B. halotolerans-nakamurai-tequilensis* (Figs 1 and S1). Among these strains, many containing YxaL1 have been observed to be primarily associated with plants and soil [13]. The beta-propeller protein BamB in *Mycobacteroides abscessus* subsp. *massiliense* is also included in the YxaL1 clade, indicating that the beta-propeller domain of BamB is commonly adopted in bacteria [4]. The consensus sequences of YxaL1 and YxaL2 were predicted to have the 8-blade propellers based on the PQQ motifs, AX(D/N)XXTG(D/E/K)XXW (Table 1; see S1 File). YxaL1 and YxaL2 are distantly related to the other paralogs that are branched to from two beta-propeller domains (ingroup/outgroup) of *E. coli* BamB and YncE, which include the only beta-propeller protein Rv1057 in *Mycobacterium tuberculosis*.

Constitutive expression and secretion of YxaL in the medium of *B. velezensis* strain GH1-13

When cultivating strain GH1-13, constitutive expression of the *yxaL* gene was observed in the cell culture supernatants at different incubation times: early-exponential (5 h), late-exponential (8 h), early-stationary (12 h), and stationary (24 h) phases of growth (Fig 2A and 2B). The quantitative RT-PCR results for the *yxaL* gene transcript levels showed a similar pattern with the transcriptomic data in the NCBI BioProject PRJNA445855. Using genomic DNA of strain GH1-13 as a template, a DNA fragment coding for the mature YxaL protein (45–415 a.a.) was amplified by PCR and cloned into the plasmid pProEX-HTA with an *N*-His tag linked to the TEV protease cleavage site to develop a simple method of producing a recombinant protein (see S2 Fig). Briefly, the recombinant protein YxaL (*N*-His-TEV) was overproduced in *E. coli*

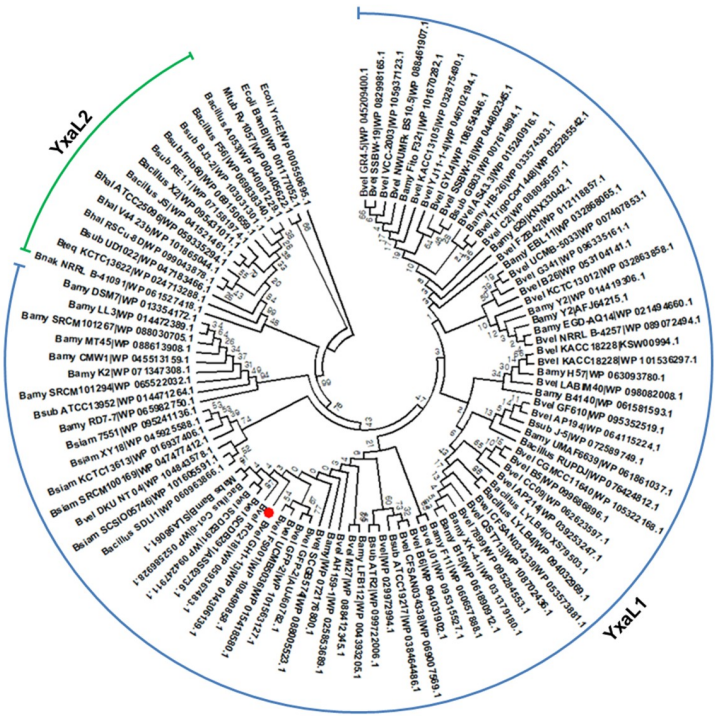

Fig 1. Phylogenetic analysis of homologous YxaL protein sequences. A minimum evolutionary tree was constructed using the Close-Neighbor-Interchange algorithm at a search level of 1, based on the Dayhoff model with 1000 bootstrap replications in MEGA 7. The Neighbor-Joining algorithm was used to generate the initial tree. The analysis involved 100 amino acid sequences. All ambiguous positions (gaps) generated from a multiple sequence alignment using Clustal Omega were removed for each sequence pair. There were a total of 630 positions in the final dataset and the evolutionary tree was drawn with branch lengths, as shown in S1 Fig. The position of YxaL obtained from *Bacillus velezensis* strain GH1-13 is marked by a circle symbol before prefixing the strain name with the abbreviated taxonomic name. Abbreviations used: Bamy, *B. amyloliquefaciens*; Bhal, *B. halotolerans*; Bnak, *B. nakamurai*; Bsiam, *B. siamensis*; Bsub, *B. subtilis*; Bteq, *B. tequilensis*; Bvel, *B. velezensis*; Ecoli, *Escherichia coli*; Mabs, *Mycobacteroides abscessus* subsp. *massiliense*; and Mtub, *Mycobacterium tuberculosis*.

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and purified repeatedly using Ni NTA affinity chromatography before and after digestion with TEV protease (*N*-His) to remove the *N*-His-TEV site from the recombinant protein.

Using this method, we achieved a high yield of 25 mg YxaL from 1 L LB medium cell culture, which was harvested 1 h after induction with 0.2 mM IPTG at the optical density (OD600) of 0.5. Size exclusion chromatography showed that purified YxaL is a monomer with a molecular mass of 39,784. Using purified YxaL as an antigen, a rabbit polyclonal antibody was generated to determine the expression level and location of YxaL in the culture medium of strain GH1-13. The western blot results showed that YxaL was constitutively expressed and secreted from the cells, which produced approximately 100 μg L^{-1} YxaL in (concentrated) supernatants, with an observed half-life of 1.6 h (Fig 2C and 2D). These results confirm the previous proteome analysis of extracellular proteins in *B. subtilis* [2], and suggest that YxaL present in the *B. subtilis* species complex is constitutively expressed and excreted into the medium.

Effects of YxaL on plant seedling growth

To investigate the effect of YxaL on plant growth, *A. thaliana* seeds were treated by soaking in various concentrations of YxaL solution before planting on 0.5×MS medium solidified with 0.5% agarose, supplemented with or without 0.5 μM auxin. When the germination rate was

Table 1. Beta-propeller motifs for two types of YxaL in the *Bacillus* species complex.

PQQ motifs (bold underlined letter)	**AXDXXTGDXXW**
	–N––E–
	——K–
YxaL type 1 consensus	*B. amyloliquefaciens-siamensis-velezensis** (415 amino acid residues)
YxaL1-1	FTSTAD**G**ALK**W** (81–91)
YxaL1-2	TAYHPD**G**TVK**W** (123–133)
YxaL1-3	FI**D**KE**TG**NILT (165–175)
YxaL1-4	PTSPS**T**WTQK**W** (209–219)
YxaL1-5	**A**I**N**SG**TG**QVK**W** (250–260)
YxaL1-6	YAYTS**TG**AVK**W** (290–300)
YxaL1-7	FSISK**TG**NMN**W** (330–340)
YxaL1-8	YAVDAD**G**NEK**W** (370–380)
YxaL type 2 consensus	*B. halotolerans-nakamurai-tequilensis* (394 amino acid residues)
YxaL2-1	FAGNTD**G**TLK**W** (72–82)
YxaL2-2	KAFNPD**G**SVK**W** (114–124)
YxaL2-3	FI**D**KE**TGE**ILT (156–166)
YxaL2-4	PTSKS**T**WTER**W** (200–210)
YxaL2-5	**A**I**N**SG**TG**QVK**W** (241–251)
YxaL2-6	YAYTS**TG**SLK**W** (281–291)
YxaL2-7	FSISKN**GD**MN**W** (321–331)
YxaL2-8	YAADAN**G**NEL**W** (361–371)

*YxaL type 1 includes *Mycobacteroides abscessus* subsp. *massiliense* BamB (NCBI accession no. SLA98061.1). Consensus sequences of YxaL1 and YxaL2 are given in S1 File.

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evaluated 2 days after planting by counting germinated seeds under a microscope, the germination rate of YxaL-treated seeds was not different as compared to that of the corresponding untreated control group on either auxin-supplemented or unsupplemented media, although the germination of seeds on auxin-supplemented media was lower than those on unsupplemented media (Fig 3A). This is congruent with previous studies [21–23], suggesting that auxin plays a crucial role in seed dormancy. In contrast, YxaL-treated seeds exhibited similar lengths after 1 week of growth of seedling roots, and were longer than those of the untreated seedling roots, both in the auxin-supplemented and unsupplemented media (Fig 3B). Furthermore, seedlings of treated seeds had markedly increased numbers of lateral roots, compared to those of untreated seeds (Fig 3C). A similar effect of YxaL on the root growth and development was also observed for rice seedlings (see S3 Fig). From these results, the optimal concentration of YxaL in the soaking solution was determined to be 1 mg L^{-1} YxaL.

Using RNA extracts from 1-week-old *Arabidopsis* roots, RT-PCR was performed to assess changes in relative expression (ΔΔCq) of genes encoding auxin-responsive protein (IAA1), indole-3-acetic acid amino synthetase (GH3.3), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS11), and abscisic acid (ABA)-responsive element binding factor (AFB4), which are responsive to auxin, ethylene, and ABA in the roots of *A. thaliana* at the early and later seedling stages [17]. The qPCR results showed that seed soaking in a low concentration of YxaL (10 to 100 μg L^{-1}) resulted in markedly increased levels of ACS11 expression in the 1-week cultured roots by 34.5 fold within a range of 6.8 to 68 folds, compared to the untreated seedling roots, while they decreased expression of IAA1, GH3.3 and ABF4 (Fig 3D). These changes were similar to those observed in auxin-supplemented media. Moreover, when YxaL-

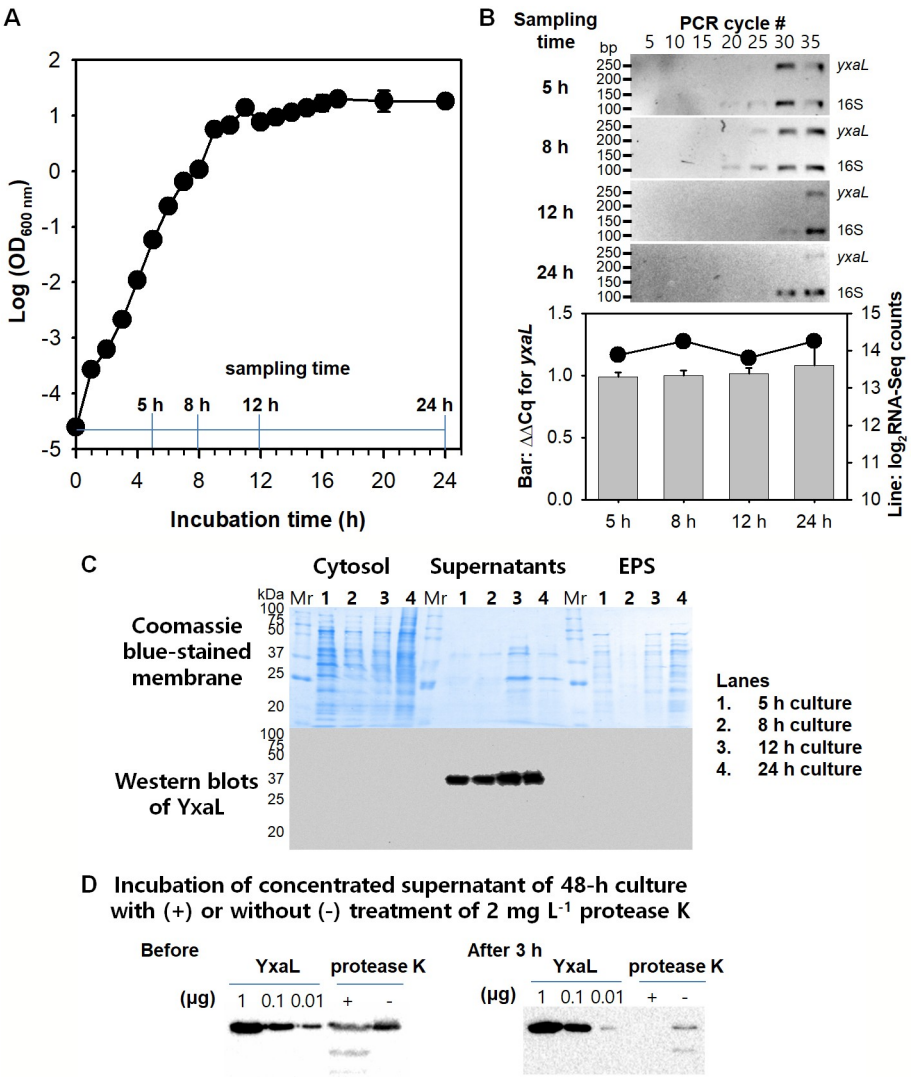

Fig 2. Constitutive expression and secretion of YxaL during cultivation of *B. velezensis* strain GH1-13. A: Growth curve of strain GH1-13 in tryptic soy broth with agitation (180 rpm) at room temperature (25˚C). Sampling time points for early-exponential (5 h), late-exponential (8 h), early-stationary (12 h), and stationary (24 h) phases of the curve are shown in the graph. B: Relative transcript (cDNA) levels of the *yxaL* gene to the 16S rRNA gene at the indicated sampling times for different growth phases of strain GH1-13. The upper panels show inverted gel images of semi-quantitative RT-PCR for expression levels of *yxaL* and 16S rRNA genes, which were evaluated every 5 cycles with a mixture of qPCR primers (S1 Table), and the lower graph shows the relative expression levels (ΔΔCq) of the *yxaL* gene to the 16S rRNA gene (bars) compared with the normalized log2-transformed values of the RNA-Seq counts obtained from the transcriptomic data deposited in the NCBI BioProject PRJNA445855. C: SDS PAGE and western blot analyses for determining the localization of YxaL in cytosol, supernatant, and extracellular matrix substance (EPS) fractions of the culture medium of strain GH1-13. The lower panels show the sensitivity and specificity of a polyclonal antibody generated using purified YxaL as the antigen (0.01 to 1 μg per lane) to determine the concentration and half-life of YxaL in (concentrated) supernatants of 48-h culture medium before and after a 3 h incubation with or without the addition of 2 mg L^{-1} protease K at room temperature.

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treated seeds were cultivated in auxin-supplemented media, they exhibited stronger inhibition of GH3.3 and AFB4 gene expression than was observed for IAA1. The altered expression levels of the above four genes indicate that YxaL has a significant effect on plant growth and development signaling pathways other than auxin.

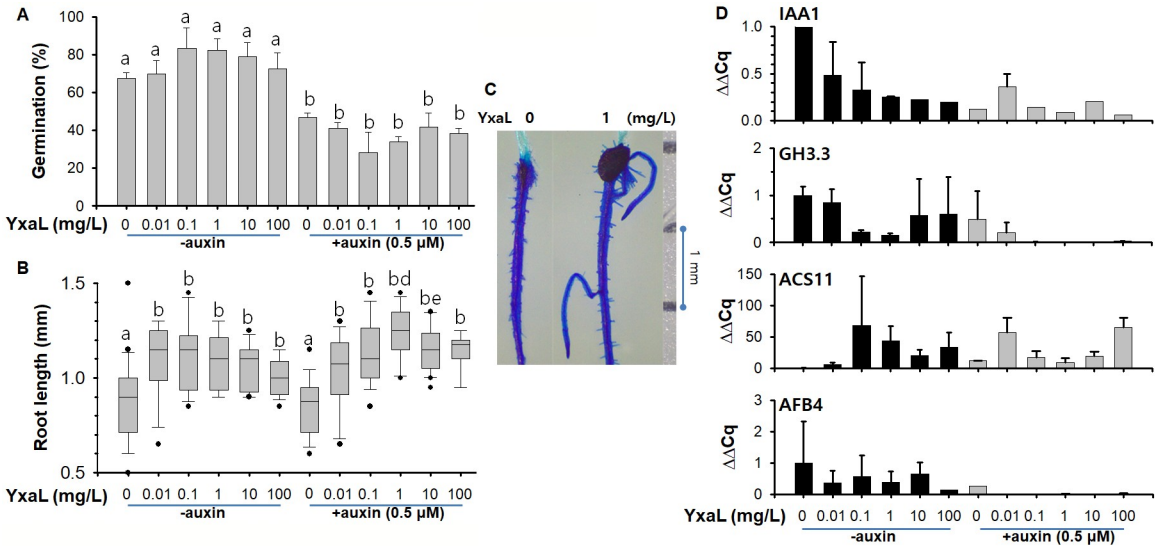

Fig 3. Differences in germination (A), root growth (B and C), and gene expression patterns (D) between YxaL-treated and untreated seeds of *A. thaliana* during seedling growth. A: Germination (%) of seeds ($n > 100$ for each group) treated with various concentrations of purified YxaL in soaking solutions and planted in auxin-supplemented and unsupplemented media. Germinated seeds were counted 2 days after planting. Compared to the untreated seeds ('a') in unsupplemented medium, significant differences were determined by two-tailed *t*-tests with a *P*-value of less than 0.05, as indicated by the letter 'b' above the error bar. B: Root lengths of 1-week cultured seedlings ($n > 30$ for each group) in auxin-supplemented and unsupplemented media after seeds were treated with various concentrations of purified YxaL in the soaking solution. Significant differences determined by two-tailed *t*-tests with a *P*-value of less than 0.05 are shown above the box plot error bar with the letter 'b' to denote the comparison with the untreated seeds ('a') and followed with the letter 'd' or 'e' to denote comparisons between paired groups for seed treatment with 1 or 10 mg L^{-1} YxaL solution before planting in auxin-supplemented and unsupplemented media. C: Comparison of root architecture between 1-week seedlings of YxaL-treated and untreated seeds in unsupplemented media. The root area is stained with methylene blue and a scale bar with 1-mm intervals is shown at the right. D: Relative expression levels (ΔΔCq) of genes coding for auxin-responsive protein (IAA1), indole-3-acetic acid amino synthetase (GH3.3), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS11), and abscisic acid (ABA)-responsive element binding factor (AFB4) in 1-week-old seedling roots grown in auxin-supplemented and unsupplemented media after seed treatment with various concentrations of purified YxaL in the soaking solution. The results, which were obtained from triplicate culture experiments with a sample size (*n*) of greater than 30 for each group of 1-week-old roots, are reported as the means and standard deviation (error bar).

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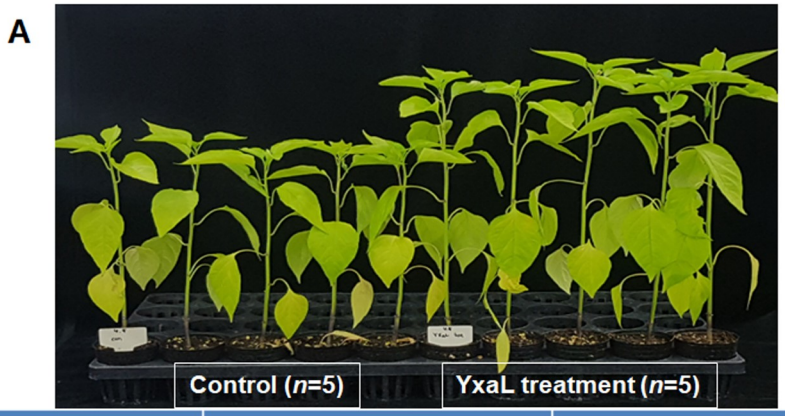
YxaL testing for plant growth and drought stress tolerance

The observed root morphology of YxaL-treated *Arabidopsis* and rice indicated that YxaL might have a common effect on the root development and growth, at a late stage, across a wide range of higher plant types, as both eudicot and monocot plants were affected here. The root system architecture response has been proposed to account not only for the plant growth [24], but also for the functionality of plasticity in root development for tolerance to a range of abiotic stresses, including nutrient limitation, drought, salinity, flooding, and extreme temperatures [25]. We observed that, when YxaL-treated and untreated seeds of pepper (*C. annuum*) were cultivated in potted soil under normal watering conditions for 54 days, YxaL-treated seeds had greater growth rates than the untreated seeds (Fig 4A). Stem heights and fresh weights of seedlings were significantly different between the two groups (two-tailed *t*-tests: $P < 0.05$), but no statistically significant difference existed between the weights of the seedling roots ($P = 0.42$), though the weights of YxaL-treated seedling roots were slightly higher than those of the untreated seedling.

Furthermore, YxaL-treated seedlings exhibited improved tolerance to drought with greater potential than the untreated seedlings to recover the growth of leaves after re-watering (Fig 4B). After drought stress was applied for 5 days, followed by re-watering for 7 days, a full



Growth rates	Untreated seeds (n=5)	YxaL treated seeds (n=5)
Stem height (cm)	13.9±0.5	15.4±0.3*
Fresh weight (g)	2.37±0.07	2.98±0.15*
Wet root weight (g)	0.37±0.04	0.42±0.05

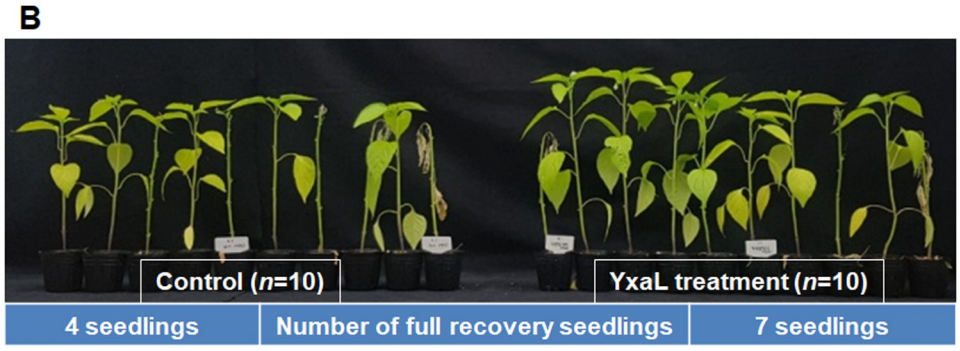


Fig 4. Effects of Yxal-treated pepper seeds on seedling growth and tolerance against drought stress. A: Shoot growth of pepper seedlings planted in potted soil after treatment of seeds in the soaking solution with or without 1 mg L^{-1} YxaL and grown in a greenhouse under normal watering conditions for 54 days. The means ± standard deviations of the measured data for stem height, fresh weight, and wet root weight were shown below the picture. Statistically significant differences between the data of the YxaL-treated and untreated groups were determined by two-tailed *t*-tests with the *P*-value of less than 0.05, as denoted by asterisks. B: Recovery of leaf status from drought stress-treated pepper seedlings of YxaL-treated and untreated seeds. After 58 days of culture in potted soil under normal watering conditions, drought stress was applied for 5 days, followed by re-watering for 7 days in order to count the number of the full recovery seedlings in each group of 10 seedlings, as noted below.

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recovery of leaf status was observed in 7 seedlings of YxaL-treated seeds in a group of 10 seedlings, while at the same time only 4 seedlings of the untreated seeds in another group of 10 seedlings recovered full leaf turgor and resumed growth. These results indicate that YxaL-treated pepper seeds are effective in reducing the leaf damage caused by drought stress, seemingly with alterations in root architecture during the seedling stage. The results obtained from this study suggest that the development and architecture of roots from YxaL-treated seeds may have potential to increase plant growth and tolerance to drought stress.

Discussion

We noted that the conserved sequences of YxaL in the plant growth-promoting *Bacillus* species complex are distantly related to the 8-blade beta-propeller protein BamB, which is essential in assembling outer membrane proteins in *E. coli* [26, 27]. This beta-propeller protein family

may have diverse functions outside of the cell, as exemplified by the only beta-propeller protein Rv1057 in *M*. *tuberculosis*, which plays a role in the secretion of the major virulence factor ESAT-6 and contributes to cytotoxicity in infected macrophages [28]. The evolutionary tree shows that *E*. *coli* YncE is a distant outgroup with low sequence similarity to other proteins. Analyses of large-scale genome and outer membrane vesicle proteome data support the possibility that a highly immunogenic YncE is broadly expressed and secreted by different pathotypes of *E*. *coli* [29, 30]. Although secreted beta-propeller proteins in bacteria are largely unexplored, mounting data suggest that they may be involved in maintenance of the cell envelope, secretion, adhesion, and host immune responses.

In this study, the mature YxaL protein was deduced from the genomic DNA of *B*. *velezensis* strain GH1-13 and utilized for recombinant protein production and purification. Using the purified YxaL protein as an antigen, a polyclonal antibody was produced to determine the constitutive expression of secreted YxaL in supernatants by western blotting, the results of which were consistent with the transcriptomic and RT-PCR data. From the plant growth experiments, *A*. *thaliana* and rice seeds soaked in a solution containing YxaL in the sub-nanomolar range (0.1 ~ 1 mg L^{-1}) showed a significant improvement with respect to root growth as did treatment with *B*. *velezensis* GH1-13 cells [9]. A previous study showed that the use of bovine serum albumin (BSA) as an organic N-source supplement produced similar effects on the root growth and development of *A*. *thaliana* at an optimal concentration of 1 g L^{-1} in the medium [31]. The effective concentration of BSA used in the medium was much higher than that of YxaL in a soaking solution which was useful to support plant growth-promoting activity for *A*. *thaliana*, rice, and pepper by the seed soaking for 2 h before planting.

The RT-PCR results for relative gene expression of phytohormone-responsive genes (IAA1, GH3.3, ACS11, and AFB4) in 1-week-old *A*. *thaliana* roots demonstrated that YxaL has a significant effect on the upregulation of ACS11, which is responsible for the synthesis of ACC, the precursor of ethylene. However, the YxaL treatment decreased the expression of the other assayed genes (IAA1, GH3.3, and AFB4), which respond to auxin and ABA. It has been well established that the expression of ACS11 is induced in all cell types of the cell division zone in response to auxin and other stresses [32]. If the ACS11 gene is under the control of Aux/IAA-auxin response factors, it should be activated by the proteolytic degradation of the Aux/IAA transcriptional repressors in response to a specific signaling cue or by the binding of YxaL to dividing cells during seedling growth and not just during the germination stage [33]. The above gene expression pattern in the roots of 1-week-old *A*. *thaliana* seedlings, which germinated from seeds soaked in a solution containing YxaL, was somewhat similar to that seen in the auxin-supplemented media. It seems likely that exogenous auxin also enhances the expression of ACS11 in dividing cells [32], whereas it reduces germination rate in seeds, whether they are treated or not with YxaL. A kinetic analysis of the auxin transcriptome during lateral root formation in *Arabidopsis* also showed that the ACS11 expression was not detectable until after auxin treatment [34]. In contrast, the YxaL treatment of seeds did not affect germination and improved the root development and seedling growth after germination, even in the presence of exogenous auxin. Considering the effects of YxaL on lateral root formation with the upregulation of ACS11 and the downregulation of the other assayed genes, it is likely that YxaL elevates ethylene synthesis and alters auxin transport leading to suboptimal levels of auxin for lateral root initiation [35, 36]. These events may involve a pathway in which YxaL may interact with auxin response factors in a vast array of auxin signaling pathways, including activation of ACS11 during seedling growth, although the regulatory mechanism is unknown.

ACC synthesis can help plants to not only synthesize ethylene, which antagonizes auxin and ABA to form branch roots, but also regulate plant responses to adverse environmental conditions by the translocation of ACC and ethylene signaling [35–37]. As we have seen that

YxaL treatment of pepper seeds was effective at increasing the seedling growth and tolerance to drought stress, YxaL might play a pivotal role in ethylene responses of the young seedling to improve plant growth and stress tolerance, as shown in *Arabidopsis* and crop species [38, 39]. Furthermore, plant-produced ACC can attract plant growth-promoting rhizobacteria (PGPR) with ACC deaminases that degrade ACC. The ACC deaminase-producing *Rhizobium leguminosarum* biovar *viciae* 128C53K promotes nodulation of pea plants, likely by modulating ethylene levels that inhibit the nodulation process in legumes [40]. In contrast, ACC deaminases of free-living PGPR are likely to increase root colonization, plant growth, and resistance to a variety of environmental stresses [41–47]. However, it is still unclear that ACC deaminase-producing PGPR decrease ethylene production in plants or produce other beneficial effects for plant growth promotion.

Numerous PGPR strains can produce a variety of active compounds, including the five major phytohormones (auxin, cytokinins, gibberellic acid, ethylene, and ABA), which directly affect plant growth and resistance through different mechanisms [48]. Besides phytohormones, volatile organic compounds (e.g., 2,3-butanediol) and antimicrobial compounds (e.g., thuricin 17), which induce systemic resistance or inhibit pathogens, also benefit especially from PGPR *Bacillus* strains [49, 50]. Expanding the area of plant-microbe interactions, our work provided insights into the role of a non-essential gene (*yxaL*) that is highly conserved in the operational group of *B. amyloliquefaciens-siamensis-velezensis* thought to be primarily related to the PGPR effects on plants [13]. Our data showed that the constitutively secreted protein YxaL is released from *B. velezensis* strain GH1-13 and is associated with the beneficial effects of PGPR on plants. To our knowledge, this is the first report that an excreted protein from PGPR is effective in helping to promote lateral root formation, likely by affecting ethylene synthesis and signaling pathways in different tissues of eudicot and monocot plants. This effect may in turn affect plant growth and drought stress resistance, as observed with seedling growth under axenic and in pot conditions after treatment of *A. thaliana*, rice, and pepper seeds in a soaking solution with purified YxaL. Our observations suggest that YxaL is a new biomaterial with potential applications for promoting plant growth and improving stress tolerance.

Conclusion

B. velezensis strain GH1-13 constitutively expressed and secreted a highly conserved beta-propeller protein YxaL in the taxonomic operational group of *B. amyloliquefaciens-velezensis-siamensis*, which is thought to be primarily associated with soil and plants. The mature YxaL protein overexpressed in *E. coli* was effectively used in the treatment of plant seeds in a soaking solution in order to promote the root development and seedling growth of different plant species. Horticulture experiments following the treatment of pepper seeds with 1 mg L^{-1} YxaL in a soaking solution were performed to address the effect of YxaL treatment with relationship to alterations in the root system architecture. These results demonstrated that YxaL treatment of plant seeds have the potential to increase plant yield and tolerance against drought stress. The application of these capacities of the PGPR *Bacillus* stains to culture media and agricultural soils may vary across time of the microbial population growth, and limited quantities are available due to the protein degradation. This paper introduced a methodology of protein overexpression and purification, which can be used not only to characterize and optimize the performance of YxaL in seed treatment use, but also to produce a modified YxaL form and a specific antibody targeted to YxaL for the structural and functional studies that are advancing our understanding of how YxaL interacts with different plant cells or tissues in different conditions and at rhizosphere levels. The invented YxaL (*N*-His) and IgG antibody products are

available for discovery of potential targets of YxaL and for microscale imaging of their dynamic changes accompanying plant gene expression related to the root development, seedling growth, and stress tolerance under various environmental conditions. This study provides a new biomaterial for plant growth promotion and stress resistance with the relation to the root growth and developments that are potentially of interest in further research and application.

Supporting information

S1 Fig. Evolutionary relationship of YxaL homologs. The evolutionary history was inferred using the minimum evolution method in MEGA 7. The tree out of 46 minimum evolution trees (sum of branch length = 5.46420670) is shown, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.
(DOCX)

S2 Fig. Recombinant protein production and purification of the mature YxaL protein using an Ni NTA agarose column. The recombinant protein YxaL with the *N*-His TEV cleavage site was purified by stepwise elution with high concentrations of imidazole (50 to 250 mM) and, after the *N*-His TEV cleavage site was removed from the recombinant protein YxaL by overnight digestion with a 1:100 ratio of a recombinant TEV protease (*N*-His), the mature protein YxaL was recovered in unbound fraction eluted by low concentrations of imidazole (20 to 25 mM) and the protein size and purity was determined by size exclusion chromatography and SDS PAGE.
(DOCX)

S3 Fig. Effects of soaking seeds with YxaL on the root growth and development of rice (*Oryza sativa* L.). After planting of rice seeds soaked with various concentrations of YxaL (0 to 100 mg L^{-1}), the primary and hair root lengths were measured on 1 week after seedling emergence.
(DOCX)

S1 File. Consensus amino acid sequences of YxaL homologous proteins. 97 homologous sequences of YxaL and 3 paralogous sequences of *Mycobacterium tuberculosis* Rv1057, *Escherichia coli* BamB and YncE were collected from the National Center for Biotechnology Information to analyse the phylogenetic trees (Figs 1 and S1) and to search the beta-propeller motif sequences (Table 1) in the consensus sequences of YxaL1 and YxaL2 generated by multiple sequence alignments.
(DOCX)

S1 Table. Primers used in qPCR for determination of the expression levels of the *Bacillus yxaL* gene and *Arabidopsis* genes. See Figs 2 and 3 for the relative expression levels of the *yxaL* gene in *Bacillus velezensis* strain GH1-13 and plant hormone-responsive marker genes (IAA1, GH3.3, AFB4, and ACS11) in *Arabidopsis thaliana* normalized to the respective 16S and 18S rRNA levels by the ΔΔCq method.
(DOCX)

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