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Stimulation of plant growth and biocontrol by *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 engineered for improved action

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Abstract

During the last decade, the use of plant-root colonizing bacteria with plant growth-promoting activity has been proven as an efficient and environmental-friendly alternative to chemical pesticides and fertilizers. Biofertilizer and biocontrol formulations prepared from endospore-forming *Bacillus* strains are increasingly applied due to their long shelf life, which is comparable with that of agrochemicals. Today, spore suspensions from natural representatives of mainly *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus pumilus* are available. However, these biofertilizers, directly prepared from environmental strains, are sometimes hampered in their action and do not fulfill in each case the expectations of the appliers (Borriss R, Bacteria in agrobiology: plant growth responses, Springer, 2011, pp. 41-76). This review will focus on several ways to improve the action of *B. amyloliquefaciens* subsp. *plantarum* FZB42^{T}, the type strain for the group of plant-associated *B. amyloliquefaciens* strains. We are focusing here on genomics and genetic engineering techniques as helpful tools for developing more powerful biofertilizer and biocontrol agents.

Keywords: Plant growth promotion; *Bacillus amyloliquefaciens* subsp. *plantarum*; Biofertilizer; Biocontrol; Harpin genes

Introduction

In recent years, use of biologicals in plant protection is steadily increasing and begins to replace, in part, chemical pesticides. An increasing number of farmers are recognizing the need for other avenues for pest control that are not as damaging to the environment and the land. Henceforth, they are turning to biopesticides to prevent pest damage in a more ecological-friendly manner that includes targeted applications, lower residues, and fewer applications. According to a comprehensive study of BCC Research, global markets for biopesticides will grow from US$54.8 billion in 2013 to US$61.8 billion in 2014. This is estimated to reach US$83.7 billion by 2019, with a 5-year compound annual growth rate (CAGR) of 6.3% from 2014 through 2019 (http://www.bccresearch.com/market-research/chemicals/biopesticides-chm029e.html). Thereby, biological preparations from spore-forming *Bacillus* spp. are preferred, because their long-term viability facilitates the development of commercial products. Unfortunately, their success in agricultural application is still hampered by insufficient knowledge about basic mechanisms of interactions between bacilli and plants, although some progress has been made in last decade [1].

Plant-associated *Bacillus amyloliquefaciens* strains belonging to subsp. *plantarum* [2] are distinguished from other representatives of endospore-forming *B. amyloliquefaciens* by their ability to colonize plant rhizosphere, to stimulate plant growth, and to suppress competing phytopathogenic bacteria and fungi. Due to their biofertilizer and biocontrol properties, they are becoming increasingly important as a natural alternative to chemical pesticides and other agrochemicals. We have focused our research on *B. amyloliquefaciens* FZB42^{T} [3], the type strain for *B. amyloliquefaciens* subsp. *plantarum*. Comparative genome analysis, transposon mutagenesis, and transcriptome and proteome analysis of this model organism are valuable means to evaluate its plant growth-promoting activity. A network of research activities was established in frame of national and international

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programs to elucidate the interaction of the beneficial bacterium with plants, plant pathogens, and the microbial community living on plant roots. The outcome of this research will contribute to the development of an efficient and environmental-friendly plant protection agent. In order to reveal the specific genomic features linked with the properties beneficial for plant growth and biocontrol, we have sequenced the whole genome of FZB42 as the first example of gram-positive plant beneficial bacteria [4].

Review

Rhizosphere bacilli

Land plants and bacteria have shared the same environment for approximately 360 to 480 million years [5]. The contact between them has developed into various dependencies on both sides. Plants and certain rhizobacteria form mutually beneficial associations mediated through an exchange of chemical metabolites [1].

Plant growth-promoting *Bacilli*, like FZB42, are able to propagate in the rhizosphere, the soil environment influenced by plant roots. This environment is highly competitive due to the nutrient-rich rhizodeposits consisting of a wide variety of compounds derived from sloughed off root cells and tissues, mucilages, and soluble exudates originating from intact roots [6]. However, roots respond to signals that stimulate defense responses (salicylic and jasmonic acids) by exuding a range of secondary metabolites, such as saponins, glucosinolates, hydroxamic acids, and naphthoquinones, which are inhibiting the growth of many 'ordinary' (not adapted to plant colonization) bacteria or fungi in this area.

The ability of FZB42 to colonize the rhizoplane is a precondition for plant growth promotion [7]. Using a GFP-tagged derivative [8], the fate of bacterial root colonization was recently studied. It ruled out that the bacterium behaves distinctly in colonizing root surfaces of different plants. In contrast to maize, FZB42 colonized preferentially root tips when colonizing *Arabidopsis thaliana* [9]. On duckweed, *Lemna minor*, FZB42 accumulated preferably along the grooves between epidermal cells of roots and in the concave spaces on ventral sides of fronds. *In vitro* studies performed with maize seedlings revealed that the segment within 2 to 8 cm distance from the basal site of the primary root was the most colonized region by FZB42. On the contrary, few bacterial cells could be observed within the range of 2 cm of root tip. In general, the green fluorescent FZB42 were decreasingly observed from the upper part of a root down to the root tip. Scanning electron microscopy confirmed the presence of FZB42 on root hairs, where the bacterial cells were usually associated with a wealth of presumed root exudates [10]. In lettuce (*Lactuca sativa*) seedlings, bacterial colonization occurred mainly on the primary roots and root hairs, as well as on root tips and adjacent border cells. Occurrence of labelled bacteria decreased towards the root tips of the lateral roots, and no colonization of the finer roots was observed [11].

Genomics and other -omics techniques are useful for dissecting interactions between FZB42 and plants

The whole genome sequence of the type strain of plant-associated *B. amyloliquefaciens* subsp. *plantarum*, FZB42^T, has been determined in 2007, as the first representative of gram-positive, plant growth-promoting bacteria. Its 3,918-kb genome, containing an estimated 3,695 protein coding sequences (CDS), lacks extended phage insertions, which occur ubiquitously in the related *Bacillus subtilis* 168 genome. The *B. amyloliquefaciens* genome reveals a huge potential to produce secondary metabolites, including the polyketides bacillaene, macrolactin, and difficidin. More than 8.5% of the genome is devoted to synthesizing antibiotics and siderophores by pathways not involving ribosomes [12]. A comparison of its genomic sequence with that of the *B. amyloliquefaciens*-type strain DSM7^T revealed significant differences in the genomic sequences of both strains [13]. The strains have in common 3,345 CDS residing in their core genomes; while 547 and 344 CDS were found to be unique in FZB42^T and DSM7^T, respectively. Notably, the gene clusters encoding non-ribosomal synthesis of antibacterial polyketides difficidin and macrolactin [14,15] are absent in DSM7^T. For comparison, *B. subtilis* 168^T has a similar number of CDS in common with *B. amyloliquefaciens* strains DSM7^T and FZB42^T (3,222 and 3,182 CDS, respectively). Meanwhile, besides FZB42^T, the genomes of other *B. amyloliquefaciens plantarum* strains have become available [16]. The core genome formed by 15 *B. amyloliquefaciens plantarum* genomes includes 3,151 genes, the pan-genome more than 6,000 genes, suggesting a high degree of flexibility in the genomes of such plant-associated *B. amyloliquefaciens* strains. Nevertheless, as has been shown in a previous study, the genomes of the *plantarum* subsp. are well distinguished from the non-plant-associated *amyloliquefaciens* subsp. [2]. In addition, except DSM7^T, the genomes of three other representatives of the subsp. *amyloliquefaciens* have been published, enabling a comparative genome analysis of plant root-associated and free-living soil *B. amyloliquefaciens* strains. Fifty-four genes were identified as being unique for subspecies *plantarum* and did not occur in the free-living soil bacterium *B. amyloliquefaciens* subsp. *amyloliquefaciens*, e.g., gene clusters involved in the synthesis of difficidin and macrolactin and in carbon metabolism (Table 1). Genes involved in ribosomal synthesis of several bacteriocins, such as mersacidin [17], plantazolicin [18], and amylocyclicin [19], were detected in several representatives of *B. amyloliquefaciens* subsp. *plantarum* but are not part of the *plantarum* core

Table 1 Singletons occurring in *Bacillus amyloliquefaciens* subsp. *plantarum* (15 genomes), but not in *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*

Accession number	Description	BS gene	Identity (%)	Class[a]
RBAM_001770	DinB family; cl17821 YizA	BSU10800	49	6.7
RBAM_002650	GH25 muramidase YbfG	BSU02200	83	1.1
RBAM_002660	Unknown protein			6.7
RBAM_003280	Alpha-amylase AmyE	BSU03040	87	2.2
RBAM_003370	Putative antimicrobial peptide Lci			4.3
RBAM_004550	8-*oxo*-dGTP diphosphatase	BSU04330	60	3.1
RBAM_004640	Unknown protein			6.7
RBAM_005260	Proline/betaine transporter, MFS superfamily			1.2
RBAM_005610	Cation exporter TrkA, CzcD	BSU26640	89	1.2
RBAM_005640	4-Hydroxy-tetrahydrodipicolinate synthase	NP_389559	25	2.6
RBAM_006120	Unknown protein	NP_389102	43	6.7
RBAM_008480	Phosphoenolpyruvate synthase, PPDK_N	NP_389764	30	2.2
RBAM_010040	DNA alkylation repair enzyme, COG4335 YhaZ	BSU09810	63	3.1
RBAM_012380	Uronate isomerase (glucuronate isomerase) UxaC	BSU12300	77	2.2
RBAM_012390	Symporter, sugar (glycoside-pentoside-hexuronide) transporter	BSU12310	80	2.2
RBAM_013540	Oxidoreductase, NADB_Rossmann	BSU13770	97	6.6
RBAM_014340	Macrolactin synthesis; polyketide synthase of type I			4.3
RBAM_014350	Macrolactin synthesis; polyketide synthase of type I			4.3
RBAM_014360	Macrolactin synthesis; polyketide synthase of type I			4.3
RBAM_014370	Macrolactin synthesis; polyketide synthase of type I			4.3
RBAM_014390	Macrolactin synthesis; polyketide synthase of type I			4.3
RBAM_014400	Macrolactin synthesis; polyketide synthase of type I			4.3
RBAM_014410	Macrolactin synthesis; putative penicillin binding protein			4.3
RBAM_017950	2-keto-3-deoxygluconokinase KdgK	BSU22110	30	2.2
RBAM_017960	Zinc-type alcohol dehydrogenase, Zn_ADH7 YjmD	BSU12330	77	2.2
RBAM_017970	2-keto-3-deoxygluconate-6-phosphate aldolase KdgA	BSU22100	41	6.2
RBAM_017990	D-mannonate oxidoreductase, NADB_Rossmann	BSU12350	74	2.2
RBAM_018000	Negative transcriptional regulator (LacI family) KdgR	BSU22120	34	3.4
RBAM_018100	Endo-1,4-beta-glucanase, glycoside hydrolase family 5	BSU18130	93	2.2
RBAM_018230	H+/gluconate symporter and related permeases	BSU40050	81	3.4
RBAM_019040	Hypothetical protein, DUF4025	YP_054581	50	6.7
RBAM_019290	Hypothetical protein YoaQ	BSU18700	68	4.2
RBAM_020240	Isochorismatase, cystein hydrolase	BSU26760	93	6.7
RBAM_021810	Involved in biosynthesis of extracellular polysaccharides	BSU23680	65	2.2
RBAM_021880	Metalloprotein with Zn binding site YqjT	BSU23750	76	6.7
RBAM_021970	Difficidin synthesis; modular polyketide synthase of type I			4.3
RBAM_021980	Difficidin synthesis; modular polyketide synthase of type I			4.3
RBAM_021990	Difficidin synthesis; modular polyketide synthase of type I			4.3
RBAM_022000	Difficidin synthesis; modular polyketide synthase of type I			4.3
RBAM_022010	Difficidin synthesis; modular polyketide synthase of type I			4.3
RBAM_022030	Difficidin synthesis; modular polyketide synthase of type I			4.3
RBAM_022050	Difficidin synthesis; acyl-CoA synthetase			4.3
RBAM_022060	Probable acyl carrier protein			4.3

Table 1 Singletons occurring in *Bacillus amyloliquefaciens* subsp. *plantarum* (15 genomes), but not in *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* (Continued)

RBAM_022070	Difficidin synthesis			4.3
RBAM_022090	Putative transcription terminator/antiterminator, NGN KOW	BSU01010	26	3.2
RBAM_026190	Hypothetical protein YjdF			6.7
RBAM_028450	Isochorismatase hydrolase, cysteine hydrolase			
RBAM_030020	Putative transcriptional regulator (LysR family), HTH_PBP2_LTTR YybE	BSU40670	31	3.4
RBAM_030030	Putative acetoacetate decarboxylase			
RBAM_030040	Uncharacterized oxidoreductase, ApbA ApbA_C	ykpB	23	6.6
RBAM_033310	Putative endonuclease V, DNA repair enzyme	ywqL	85	
RBAM_034390	ABC transporter permease, COG1284 (2xDUF161)	NP_388993	30	
RBAM_037270	Cupin (JmjC) domain protein, cupin 8			
RBAM_037280	ABC transporters with duplicated ATPase UuP	NP_388476	33	
RBAM_037810	Hypothetical protein 2xDUF1529			6.7

[a]Functional classes: 1.1 cellular processes/cell envelope, 1.2 cellular processes/transporters, 2.2 metabolism/carbon metabolism, 2.6 metabolism/additional metabolic pathways, 3.1 information processing/genetics, 3.2 information processing/RNA synthesis and degradation, 3.4 information processing/regulation of gene expression, 4.2 lifestyles/sporulation, 4.3 lifestyles/coping with stress, 6.2 groups of genes/membrane proteins, 6.6 groups of genes/poorly characterized/putative enzymes, 6.7 groups of genes/genes of unknown function.

genome. We conclude that most of the genes unique in subsp. *plantarum* are involved in plant-bacteria interactions. In order to support this idea, we have performed transposon mutagenesis and transcriptome and proteome analysis of FZB42 exposed directly to plants or plant root exudates [11,20,21]. Adding of root exudates up to a final concentration of 250 mg dry weight per liter of culture medium was found sufficient to cause a significant response of the FZB42 transcriptome and proteome during transient growth stage. Among the 302 genes with significantly altered expression by root exudates, 189 were annotated with known functions. The transcription of 46 genes involved in carbon and nitrogen utilization was altered in response to root exudates, with 43 of them being upregulated.

A total of 12 genes encoding enzymes involved in the Embden-Meyerhof-Parnas (EMP) pathway (including *pgi* encoding for glucose-6-phosphate isomerase) and the TCA cycle were significantly upregulated. Nearly a quarter of the genes with altered transcription (46 out of 189) were involved in uptake or utilization of nutrients. This observation corroborated that root exudates serve as energy sources in the interaction between roots and rhizobacteria. A representative selection of genes involved in plant-bacteria interaction is compiled in Table 2.

Plant growth promoting bacilli engineered for enhanced efficiency

An important feature of plant growth promoting rhizobacteria (PGPRs) is their root colonization activity [22]. After identifying genes involved in root colonization and other plant-bacteria interactions, markerless gene targeting techniques (strains without linked antibiotic resistance marker) are useful techniques in order to generate strains with enhanced rhizosphere competence. Enhanced root colonization and biocontrol activity was gained in *B. amyloliquefaciens* SQR9 by disruption of the global regulator *abrB* gene [23]. Other genes, involved in the expression of antimicrobial compounds can also be targeted. The global regulator gene *degU* was shown to control non-ribosomal synthesis of bacillomycin D [25] and bacilysin [26] in FZB42, for example.

Alternatively, reisolating of improved plant growth-promoting strains after being exposed to the natural environment for a distinct time interval, e.g., one vegetation period, is a promising approach [27]. Sequences of the unique restriction modification systems (RM) can serve as a kind of molecular ‘barcode’, facilitating specific strain identification in the environment. In contrast to *Pseudomonas fluorescens* and some other gram-negative bacteria, bacilli are known as comparable ‘weak’ colonizers of plant root surfaces, and plant growth-promoting bacilli are hardly detected later than 3 months after their application [28].

We have developed a specific method to detect FZB42 in environmental samples, previously treated with *B. amyloliquefaciens* (Rhizovital®, ABiTEP GmbH, Berlin, Germany), by combining specific methods of enrichment and molecular detection. Five months after its application in field trials, we took soil samples for reisolating FZB42 derivatives. We obtained colonies with the typical morphology of FZB42, after a complex enrichment procedure consisting of the following steps: (1) resuspending of 2.5-g sample material in 25 ml distilled water under shaking for 2 h, (2) boiling for 1 h, (3) 10 ml of the suspension was added to 40 ml liquid minimal medium with lactose as single carbon source for 1 to 2 days until rod-like bacteria became visible in

Table 2 Genes involved in plant-bacteria interactions in FZB42

Accession	Gene	Function
Synthesis of IAA (Idris et al. [24])		
RBAM_020800	*trpBA*	Tryptophan synthase subunits A and B, plant growth promotion
RBAM_020840	*trpED*	Anthranilate synthase, transferase, plant growth promotion
RBAM_035380	*ysnE*	Putative IAA acetyl transferase, plant growth promotion
Transposon mutagenesis (Budiharjo et al. [11])		
RBAM_032640	*degU*	Two-component response regulator, swarming, biofilm formation, root colonization
RBAM_030060	*yusV*	Putative iron (III) ABC transport ATPase, biofilm formation, root colonization
RBAM_035360	*nfrA*	NADPH-flavin oxidoreductase, root colonization, plant growth promotion
RBAM017410		61 aa protein, plant growth promotion
Transcriptome, enhanced in presence of root exudate (Fan et al. [20])		
RBAM_016150	*fliM*	Flagellar motor switch protein FliM, motility and chemotaxis
RBAM_016190	*fliP*	Flagellar biosynthetic protein FliP, motility and chemotaxis
RBAM_016290	*cheC*	Chemotaxis protein CheC, motility and chemotaxis
RBAM_032580	*flgM*	Negative regulator of flagellin synthesis, motility and chemotaxis
RBAM_032510	***hag***	Flagellin; involved in elicitation of plant basal defense, motility and chemotaxis
RBAM_027680	*luxS*	S-ribosylhomocysteine lyase LuxS, biofilm formation
RBAM_016860	*ymcA*	Control of community development, biofilm formation
RBAM_031630	*epsE*	Putative exopolysaccharide biosynthesis protein, biofilm formation
RBAM_001610	*secY*	Preprotein translocase subunit SecY, sec-dependent protein export
RBAM_001250	*secE*	Preprotein translocase subunit, sec-dependent protein export
RBAM_002940	*tatA*	sec-independent protein translocase protein TatAD
RBAM_002950	*tatC*	sec-independent protein translocase protein TatCD
RBAM_026150	*phoP*	Two-component response regulator, global regulation of the pho regulon
RBAM_008360	*glvA*	Maltose-6′-phosphate glucosidase GlvA, maltose metabolism
RBAM_008380	*glvC*	Phosphotransferase system (PTS) maltose-specific enzyme IICB
RBAM_008370	*glvR*	HTH-type transcriptional regulator GlvR, maltose operon
RBAM_035460	*galK*	Galactokinase GalK, galactose metabolism
RBAM_028430	*pgi*	Glucose-6-phosphate isomerase Pgi, carbon core metabolism
RBAM_006560	*ydjE*	Fructokinase homologue YdjE, carbon core metabolism
RBAM_026060	*gapB*	Glyceraldehyde-3-phosphate dehydrogenase, carbon core metabolism
RBAM_031290	*pgk*	Phosphoglycerate kinase, carbon core metabolism, gluconeogenesis
RBAM_031270	*pgm2*	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase Pgm
RBAM_008330	*acoL*	Acetoin dehydrogenase E3 (dihydrolipoamide dehydrogenase) AcoL
RBAM_014440	*pdhC*	Pyruvate dehydrogenase E2 (dihydrolipoamide acetyltransferase) PdhC
RBAM_026180	*citZ*	Citrate synthase II CitZ, carbon core metabolism, TCA cycle
RBAM_017800	*citB*	Aconitate hydratase CitB, carbon core metabolism, TCA cycle
RBAM_019120	*odhB*	Succinyltransferase of 2-oxoglutarate dehydrogenase complex
RBAM_015920	*sucC*	Succinyl-CoA synthetase (beta subunit), carbon core metabolism, TCA
RBAM_025500	*sdhB*	Succinate dehydrogenase (iron-sulfur protein), carbon core metabolism
RBAM_026160	***mdh***	Malate dehydrogenase Mdh, carbon core metabolism, TCA cycle
RBAM_035770	*licA*	PTS lichenan-specific enzyme IIA
RBAM_035760	*licH*	6-Phospho-beta-glucosidase, utilization of lichenan
RBAM_036780	*iolA*	Methylmalonate-semialdehyde dehydrogenase, utilization of inositol
RBAM_036770	*iolB*	Inositol utilization protein B (IolB)

Table 2 Genes involved in plant-bacteria interactions in FZB42 *(Continued)*

RBAM_036760	*iolC*	Inositol utilization protein C (IolC)
RBAM_036750	*iolD*	Inositol utilization protein D (IolD)
RBAM_036740	*iolE*	Inositol utilization protein E (IolE)
RBAM_036730	*iolF*	Inositol transport protein IolF
RBAM_036720	*iolG*	Myo-inositol 2-dehydrogenase IolG
RBAM_036700	*iolI*	Inositol utilization protein I (IolI)
RBAM_036800	*iolS*	Inositol utilization protein S (IolS)
RBAM_006650	*bdhA*	Acetoin reductase/butanediol dehydrogenase, synthesis of volatiles
RBAM_011430	***oppA***	Oligopeptide ABC transporter (binding protein) OppA
RBAM_011460	*oppD*	Oligopeptide ABC transporter (ATP-binding protein) OppD
RBAM_011470	*oppF*	Oligopeptide ABC transporter (ATP-binding protein) OppF
RBAM_015410	*cysP*	Sulfate permease CysP
RBAM_016930	*baeE*	Malonyl-CoA-[acyl-carrier protein] transacylase (AT) BaeE
RBAM_016970	*baeI*	Enoyl-CoA-hydratase BaeI, synthesis of bacillaene
RBAM_016990	*baeL*	Modular polyketide synthase BaeL, synthesis of bacillaene
RBAM_017010	*baeN*	Hybrid NRPS/PKS BaeN, synthesis of bacillaene
RBAM_017020	*baeR*	Modular polyketide synthase BaeR, synthesis of bacillaene
RBAM_022010	*dfnF*	Modular polyketide synthase of type I DfnF, synthesis of difficidin
RBAM_022000	*dfnG*	Modular polyketide synthase of type I DfnG, synthesis of difficidin
RBAM_021980	*dfnI*	Modular polyketide synthase of type I DfnI, synthesis of difficidin
RBAM_021970	*dfnJ*	Modular polyketide synthase of type I DfnJ, synthesis of difficidin
RBAM_014400	*mlnH*	Polyketide synthase of type I MlnH, synthesis of macrolactin
RBAM_018420	*fenE*	Fengycin synthetase FenE, synthesis of fengycin
RBAM_003680	*srfAC*	Surfactin synthetase C SrfAC, synthesis of surfactin
RBAM_003690	*srfAD*	Surfactin synthetase D SrfAD, synthesis of surfactin
Secretome enhanced in the presence of root exudate (Kierul, unpublished)		
RBAM_032500	*fliD*	Flagellin HAP2; involved in elicitation of plant basal defense, motility
RBAM_032510	***hag***	Flagellin; involved in elicitation of plant basal defense, motility and chemotaxis
RBAM_026420	*tpx*	Thiol peroxidase, resistance against oxidative stress
RBAM_020470	*ponA*	Bifunctional glucosyltransferase/transpeptidase, membrane protein
RBAM_011380	*appA*	Oligopeptide ABC transporter (binding protein)
RBAM_011430	***oppA***	Oligopeptide ABC transporter (binding protein)
RBAM_023290	*pstS*	Phosphate ABC transporter (binding protein)
RBAM_022520	*yqiG*	NADH-dependent flavin oxidoreductase
RBAM_026160	***mdh***	Malate dehydrogenase Mdh, carbon core metabolism, TCA cycle
RBAM_033170	*alsS*	Acetolactate synthase, synthesis of 2,3-butanediol, plant growth promotion, elicitation of plant ISR
RBAM_025870	*abnA*	Arabinan-endo 1,5-alpha-L-arabinase, utilization of arabinan
RBAM_017540	*chbA*	Putative chitin-binding protein, utilization of chitin/chitosan
RBAM_018140	*xynC*	Endo-1,4-beta-xylanase, utilization of xylan
RBAM_035930	*gmuG*	Endo-beta-1,4-mannanase, utilization of glucomannan

Data were compiled from Idris et al. (2007), Fan et al. [20], Budiharjo et al. [11], and Kierul et al. (in preparation). Genes involved in plant-bacteria interactions in FZB42. The genes detected in both transcriptome and secretome analysis are in bold.

the microscopic sample, and (4) plating of the 10-1 to 10-5 diluted samples onto lactose (0.1%) minimal agar plates with 0.1% azure dye-stained hydroxyl ethyl cellulose (AZCL HE). Colonies hydrolyzing AZCL and displaying the typical morphology of FZB42 (rough, flat, dendritic, translucent, white) were analyzed after 3 days for presence

Table 3 Characterization of isolates from soil samples obtained 5 months after application of FZB42 to *Antirrhinum majus* cultures in October 2009, Chengong County, Kunming

Strain	Lactose MM	Cellulose	RM (785 bp)	Nrs (839 bp)	Pzn (821 bp)	Morphology (nutrient agar)
FZB42	+	+	+	+	+	Rough, flat, dendritic, translucent, white
KM 1-1	+	+	+	+	+	As FZB42
KM 1-2	+	+	+	+	+	As FZB42
KM 1-3	+	+	+	+	+	As FZB42
KM 2A	+	+	+	+	+	As FZB42
KM 2B	+	+	+	+	+	As FZB42
KM3	+	+	+	+	+	As FZB42
KM 4-1	+	+	+	+	+	As FZB42
KM 4-2	+	+	+	+	+	As FZB42
KM 5-1	+	+	+	+	+	As FZB42
KM 5-2	+	+	+	+	+	As FZB42
KM 6-1	+	+	+	+	+	As FZB42
KM 6-2	+	+	+	+	+	As FZB42
DSM7^T	+	–	–	–	–	Rough, white
B. subtilis DSM10^T	–	+	–	–	–	Soft, cream

The colonies were analyzed after 3 days for presence of a unique 785-bp DNA fragment by PCR using primers PRBrm5215 and PRBrm6000 (see text). In addition, two other primer pairs PRBnrs3104 5′…tggagaaatatcactgaacaatgc and PRBnrs3943 5′…acgtttagtttcagttctttcacc for detection of the *nrs* gene cluster and PRBptn6179 5′gatagaagtattagcctggaagca and PRBptn7000 5′…tggaggaggtaacaattatgactc for detection of the *pzn* (plantazolicin) gene cluster were used. Annealing temperature of 55°C was generally used in PCR.

of an unique 785-bp DNA fragment by PCR using primers PRBrm5215 5′…TGATGGAGTAAATAATAAGGCTGG and PRBrm6000 5′… AATACATCTAAAGTTGCATC CACC. Amplification with two other primer pairs (Table 3) indicating presence of the *nrs* and *pzn* gene clusters was also found useful for examining the obtained colonies by either colony PCR or using isolated chromosomal DNA as template. The isolated colonies were examined for their ability to colonize plant roots using microbial standard techniques [7], and the genomes of the selected clones were sequenced to detect mutations possibly responsible for their improved capability to colonize plant roots. We propagated the most promising clones to obtain samples used in greenhouse and field trials. By using that approach, it was possible to obtain isolates with improved ability to colonize plant roots without engineering their genomes. Field trials to demonstrate enhanced positive response by the plant are underway.

In the following, we describe in more detail, a possibility to obtain more efficient strains by applying genetic engineering techniques in the plant growth-promoting strain FZB42. This work has been performed in the laboratory of Xuewen Gao, Nanjing Agriculture University, China. We have to acknowledge that at present, use of such engineered PGPR strains under field conditions is refused by the public, at least in Europe. However, in light of a steadily increasing world's population growing from 7 billion now to 8.3 billion in 2025 [29], innovative approaches for getting higher harvest yields without using increasing amounts of agrochemicals should not longer be excluded, given that their use is safe and without harmful consequences for human beings and nature. Careful environmental studies are a precondition before releasing genetic engineered bacteria into the environment.

Case study: expression of the Harpin gene enhances biocontrol activity of FZB42

The plant immune system has gained recognition as a major factor in the growth and development of plants and the resistance to disease, predation, and environmental stress. The *hrp* ('harp') genes encode type III secretory proteins enabling many phytopathogenic bacteria to elicit a hypersensitive response (HR) on nonhost or resistant host plants and induce pathogenesis on susceptible hosts. The HR is a rapid localized death of the host cells that occurs upon pathogen infection and, together with the expression of a complex array of defense-related genes, is a component of plant resistance. The plant genes create a cascade of effects which promote a systemic acquired resistance (SAR) throughout the plant. Beneficial effects on plant growth and health have been reported [30].

The *hrp* genes were first identified in *Pseudomonas syringae* pv. *phaseolicola*, a bean pathogen [31], and then in the plant pathogen *Erwinia amylovora* by the group of Steven Beer at Cornell University [32]. An optimized technology for producing the *E. amylovora* Harpin in a

recombinant *Escherichia coli* strain was subsequently developed [33]. Today, Plant Health Care (PHC) promotes $Harpin_{aß}$ as foliar applicant and seed treatment on the global crop market.

Xanthomonas oryzae pv. *oryzicola*, the cause of bacterial leaf streak in rice, possesses clusters of *hrp* genes that determine its ability to elicit a HR in non-host tobacco and pathogenicity in host rice [34,35]. The *hpa1* gene of *Xanthomonas oryzae* pv. *oryzae* encodes a 13-kDa glycine-rich protein with a composition similar to those of the harpins in *Xanthomonas* spp. and PopA in *Rhizoctonia solanaceum* [36]. The $hpa1_{XooC}$ gene was cloned and expressed in *E. coli* BL21 [37]. It is a member of the Harpin group of proteins, eliciting hypersensitive cell death in non-host plants, inducing disease and insect resistance in plants, and enhancing plant growth. Despite completely different sequence, its function was found very similar to that of the *E. amylovora* Harpin protein [38]. Transgenic tobacco plants expressing the $hpa1_{XooC}$ gene were constructed but were found unable to induce hypersensitive cell death (HCD) [39].

The $hpaG_{XooC}$ gene had been cloned on an expression plasmid in *B. subtilis* OKB105, a derivative of *B. subtilis* 168 which is able to produce surfactin [40] and to colonize plant roots. Application experiments in tomato plants demonstrated that OKB105 expressing $HpaG_{XooC}$ was improved in its biocontrol activity [41]. However, after 100 generations, the $HpaG_{XooC}$ expression plasmid pM43HF is unstable in *B. subtilis*, which does not allow the use of this system under large-scale conditions in practice [42]. In order to overcome this difficulty, the groups from Nanjing Agricultural University and Humboldt University decided to use the plant growth-promoting model strain FZB42 as a host for establishing a durable and efficient $HpaG_{XooC}$ expression system [43]. In order to avoid proteolytic destruction of the recombinant harpin gene product, we removed the two main extracellular proteases Apr and Npr from FZB42. Chromosomal integration of two *hpa1* genes cloned from *X. oryzae* under the control of the strong P_{43} promoter allowed stability and constitutive expression of the *hpa1* gene product in FZB42. The experiment was described extensively in [43], but unfortunately in Chinese. Here, we present a short outline.

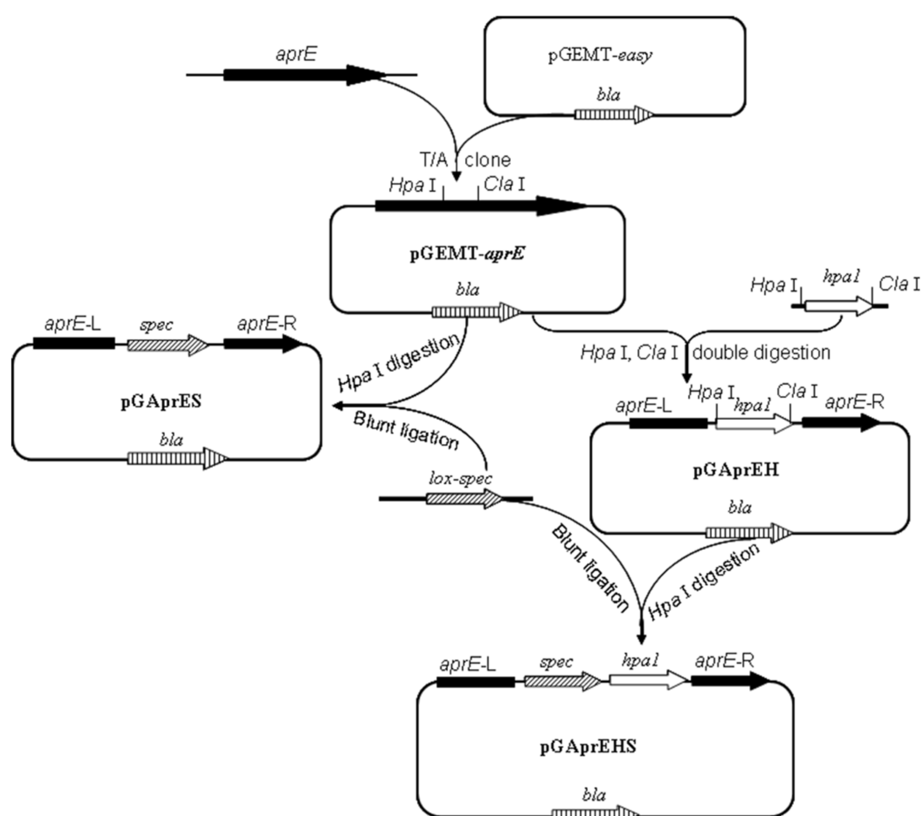

Figure 1 Construction of the Harpin expression vector pGAprEHS. The amplified partial FZB42 *aprE* gene sequence was cloned into pGEMT T/A vector resulting in pGEMT-*aprE*. Then, the gene encoding $HpaG_{XooC}$ was inserted into the double-digested (HpaI and ClaI) plasmid pGEMT-*aprE* resulting in pGAprEH. Finally, the antibiotic-resistance marker Spec was amplified from plasmid pIC333 and inserted into vector pGAprEH, yielding integration vector pGAprEHS. In addition, the *aprE* knockout vector pGAprES was obtained when the antibiotic cassette was inserted into plasmid pGEMT-*aprE*.

Briefly, two recombinant Harpin protein integration vectors, pGAprEHS (Figure 1) and pUNprEKHS (Figure 2), were constructed. The two vectors contained the powerful P_{43} promoter and the *nprB* signal peptide, which were fused with the gene encoding $HpaG_{XooC}$. In addition, the vectors, which were unable to replicate freely in *Bacillus* cells, contained parts of the *aprE* and *nprE* sequences of FZB42, allowing their target-specific integration into the FZB42 chromosome. Transformation of pGAprEHS into competent cells of FZB42 resulted in the removal of the two main proteases, AprE and NprE, and yielding FZB42AN (Figure 3A). Subsequently, two copies of the *hpa1* gene were inserted into the former protease gene sites in FZB42AN using plasmid pUNprEKHS. The resulting engineered strain FZBHarpin (Table 4) contained two copies of the *hpa1* gene and the antibiotic marker fragments (lox-Km, lox-Spec, Cre-lox).

Experimental methods

The tobacco (*Nicotiana tabacum* cv. NC89) seeds were first soaked in FZB-derived strain suspensions with a final concentration of 1×10^8 CFU for 12 h and then disinfested in a 15% (*w*/*v*) solution of sodium hypochlorite for 15 min and washed three times with sterilized distilled water. These seeds were sown onto square Petri dishes (10 cm^2) containing solidified Murashige medium. Each treatment included five plates with ten seeds each, and the experiment was replicated three times. The petri dishes were incubated in an illuminated incubator (200 $\mu E\ m^{-2}\ s^{-1}$ at 25°C) with a 16-h day and 8-h night cycle. The root length was measured after 4 to 5 weeks.

FZB42, FZBAN, and FZBHarpin were cultivated in Landy medium at 30° and 200 rpm. Bacterial cultures were taken after 24, 48, and 72 h, respectively, and were infiltrated into the intercellular space of tobacco leaves (*N. tabacum* L. 'xanthi'). The Landy medium and the Harpin protein purified from *E. coli* (50 $ug \cdot ml^{-1}$) served as the negative and positive controls, respectively. The development of the HR was registered after incubation for 24 to 36 h at room temperature.

The rice cultivar 'Fengyou 22' was used in this study. FZB-derived strains were shaken at 200 rpm at 30°C for

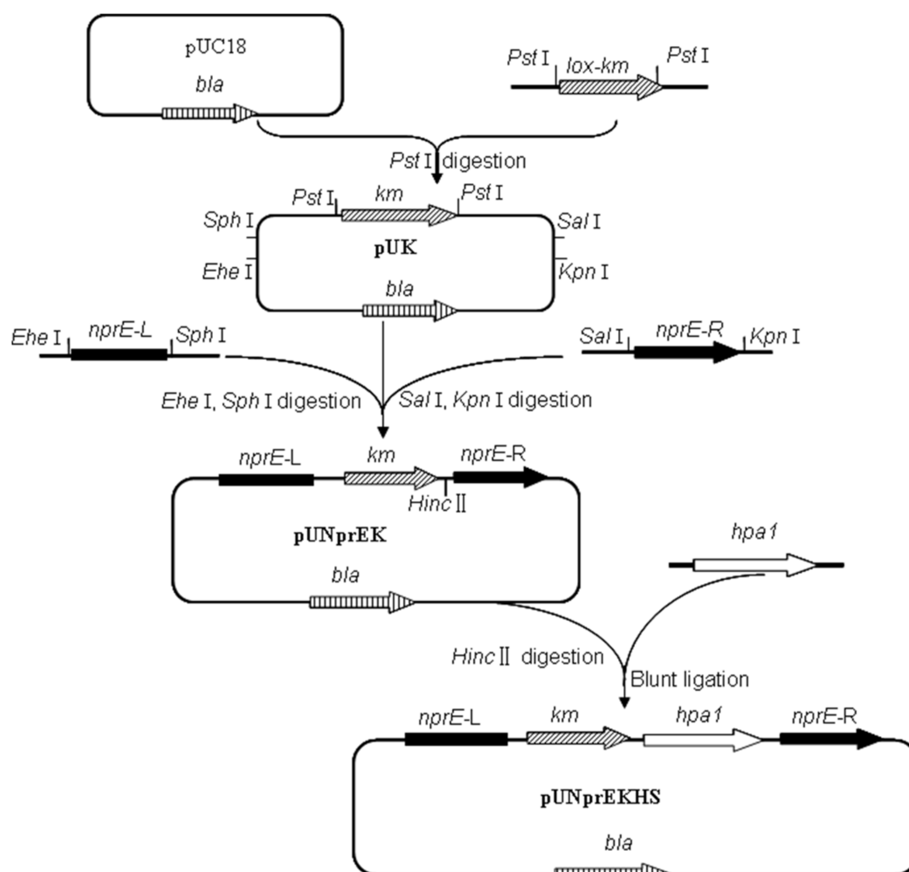

Figure 2 Construction of the Harpin expression vector pUNprEKHS. The resistance marker lox-Km was obtained from plasmid pBT2-arcA (Leibig et al. [45]) after *Pst*I digestion and subsequent cloning into pUC18. The resulting plasmid was named pUK. The upstream and downstream sequences of the *nprE* gene were inserted into plasmid pUK, yielding *nprE* knockout vector pUNprEK. The Harpin gene fused with the P_{43} promoter and the *nprE* signal peptide was inserted into the *Hinc* II site of pUNprEK, yielding pUNprEKHS.

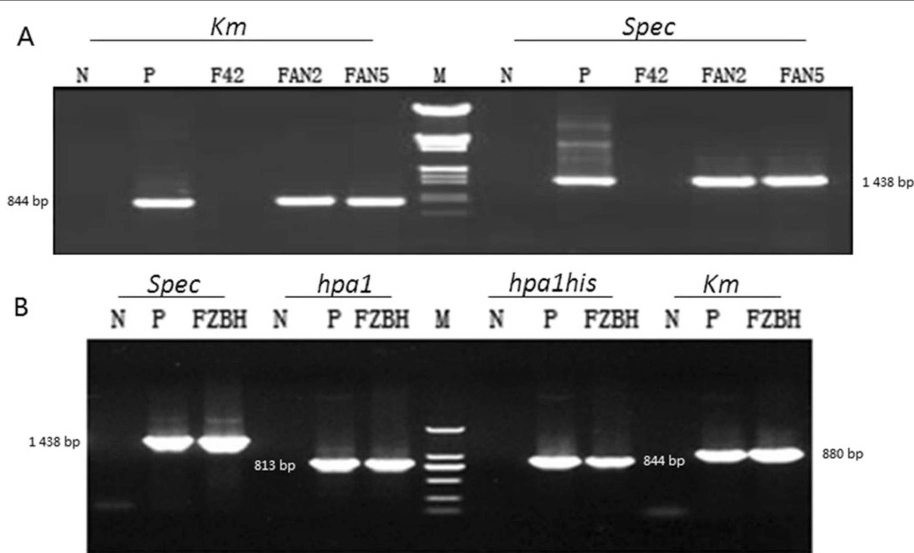

Figure 3 PCR analysis of FZBAN and FZBHarpin. (A) Validation of FZB42AN (FAN, *DnprE DnprA*, deficient in neutral and alkaline protease). Left: N, negative control (double distilled water); P, 844-bp fragment from the Km-containing plasmid; FAN 2 and FAN5, presence of the Km resistance cassette in two FZB42AN isolates. Right: N, negative control (double distilled water); P, 1,438-bp fragment from the Spec-containing plasmids; FAN 2 and FAN5, respectively, presence of the Spec resistance cassette in the two FZB42AN isolates. **(B)** Validation of FZBHarpin (FZBH, *nprE::hpa1 nprA::hpa1*). Lanes from left to right: presence of the *spec* resistance gene (1,438 bp) in FZB42Harpin, presence of the *hpa1* gene (813 bp) in FZB42Harpin, presence of the his-tagged *hpa1* gene (844 bp) in FZB42Harpin, and presence of the *km* resistance gene (880 bp) in FZB42Harpin.

72 h. The culture was adjusted to about 1×10^8 CFU ml^{-1} with sterile distilled water for use. The rice seeds were surface-sterilized using sodium hypochlorite (15%, *v*/*v*) for 15 min, washed three times in sterile water, and allowed to germinate for 2 to 3 days at 25°C. Then, the seeds were soaked in the *Bacillus* spore suspension at 25°C for 2 h. The seeds were sown into sterile soil pots, containing a mixture of vermiculite and organic manure (1:1, *w*/*w*). Five plants are placed in each pot, and each treatment includes ten pots. These pots were cultivated in a greenhouse at 18°C to 30°C. After 45 days, the FZB suspension was diluted to 10^8 CFU ml^{-1} and used for spray treatment. After 1 day, the treated pots were inoculated with the bacterial leaf blight pathogen *X. oryzae* pv. *oryzae* POX99 (ca. 10^9 CFU ml^{-1}). Bacterial leaf blight symptoms were assessed 21 days after inoculation. The height of the plants was measured.

Results and discussion

Quantitative real time PCR (qPCR) revealed constitutive expression of the two Harpin genes in the transgenic FZBHarpin strain (Figure 4). Although the harpin gene products were not detected in the supernatant of FZBHarpin by SDS-PA gel electrophoresis, their effect on tobacco plants were clearly visible: HR on tobacco was induced by supernatants taken from FZBHarpin cultures, demonstrating that biological active Harpin protein was secreted into the medium (Figure 5). Moreover, the plant growth-promoting effect of FZB42 was found to be increased in the FZB42Harpin derivative, as demonstrated by enhanced root growth. The average root length in FZB42Harpin was increased by 30% compared to the untreated control (Figure 6).

Greenhouse experiments demonstrated efficacy of FZBHarpin in biocontrolling rice bacterial blight. The control efficacy of FZB42Harpin was 51.9%. In addition, a plant growth-promoting effect by FZB42Harpin exceeding that of FZB42 was also detected (Table 5). Before applying the recombinant FZB42Harpin strain in field trials, removal of the two resistance markers flanked by the *Cre-lox* recombinase recognition sites via site-directed recombination has to be performed.

Outlook: how to improve acceptance for use of genetic engineered bacteria for enhancing crop yield?

Today, application and release of genetic engineered bacteria directly in the environment is not accepted by the public, and governmental regulations are contradictory for use of such microorganisms in enhancing crop yield. One reason is the presence of resistance genes in transgenic strains, which have been introduced in the bacteria during the allelic replacement process, and methods avoiding use of such marker genes are therefore highly desirable.

Table 4 Strains and plasmids used for constructing FZB42Harpin

Strains/ plasmids	Description	Reference or source
Plasmids		
pUC18	Cloning vector; *lacZ* Ap^{r}	Lab collection
pGEMT-easy	T/A-clone site vector; *lacZ*; Ap^{r}	Promega Corp. Fitchburg, Wisconsin
pBT2-arcA	Allelic replacement vector for *Staphylococcus aureus* containing a Km resistance cassette	Leibig et al. [45]
pIC333	A vector carrying mini-Tn10 transposase gene for *Bacillus subtilis*, offer of Spec cassette	Laboratory stock
pM43HF	Expression vector carrying *hpa1* gene under the control of promoter p43 and the *nprB* signal peptide	Wu et al. [42]
pGAprEHS	pGEM-T carrying a 2.8-kb fragment containing *aprE*, a 1.4-kb fragment *lox-Spec* and a 0.8-kb fragment *hpa1*; Ap^{r}, $Spec^{r}$	This study
pGAprES	pGEM-T carrying a 2.8-kb fragment containing *aprE*, a 1.4-kb fragment *lox-Spec*; Ap^{r}, $Spec^{r}$	This study
pUNprEKHS	pUC18 plasmid carrying a *lox-Km* cassette, *nprE*-L, *nprE*-R fragment, *hpa1-his* fragment; Ap^{R}, Km^{R}	This study
pUNprEK	pUC18 plasmid carrying a *lox-Km* cassette, *nprE*-L fragment and *nprE*-R fragment; Ap^{R}, Km^{R}	This study
Strains		
E. coli		
Topo10	*F-mcrA* Δ*(mrr-hsdRMS-mcrBC φ80lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ*(ara-leu)7697 galE15 galK16 rpsL(*Str^{R}*) endA1* λ^{-}	Invitrogen
Bacillus amyloliquefaciens		
FZB42	Type strain for *Bacillus amyloliquefaciens* subsp. *plantarum*	ABiTEP GmbH, Berlin, Germany
FZB42/AHS	FZB42△*aprE*△*nprE*:: *hpa1*:: *lox-Spec*	This study
FZBHarpin	FZB42△*aprE*△*nprE*:: *hpa1*:: *hpa1his*:: *lox-Spec*::*lox-Km*	This study
FZBAN	FZB42△*aprE*△*nprE*:: *lox-Spec*::*lox-Km*	This study
Xanthomonas oryzae pv. *oryzae* POX99		This study

Today there are several methods for marker removal available. Wang et al. [44] developed a simple and efficient *B. subtilis* genome editing method in which targeted gene(s) could be inactivated by single-stranded PCR product(s) flanked by short homology regions, and in-frame deletion could be achieved by incubating the transformants at 42°C. In this process, homologous recombination was promoted by the lambda beta protein synthesized under the control of promoter PRM in the lambda cI857 PRM-PR promoter system on a temperature-sensitive plasmid pWY121.

Alternatively, site-specific recombination systems are capable of eliminating antibiotic resistance markers, if they are flanked by recombinase recognition sites as it is the case in FZB42Harpin. In a previous study [45], a Cre-*lox* setting was established that allowed the efficient removal of resistance genes from the genomes of *Staphylococcus carnosus* and *Staphylococcus aureus*. Two cassettes conferring resistance to erythromycin or kanamycin were flanked with wild-type or mutant *lox* sites, respectively, and used to delete single genes and an entire operon. After transformation of the cells with a newly constructed *cre* expression plasmid, genomic

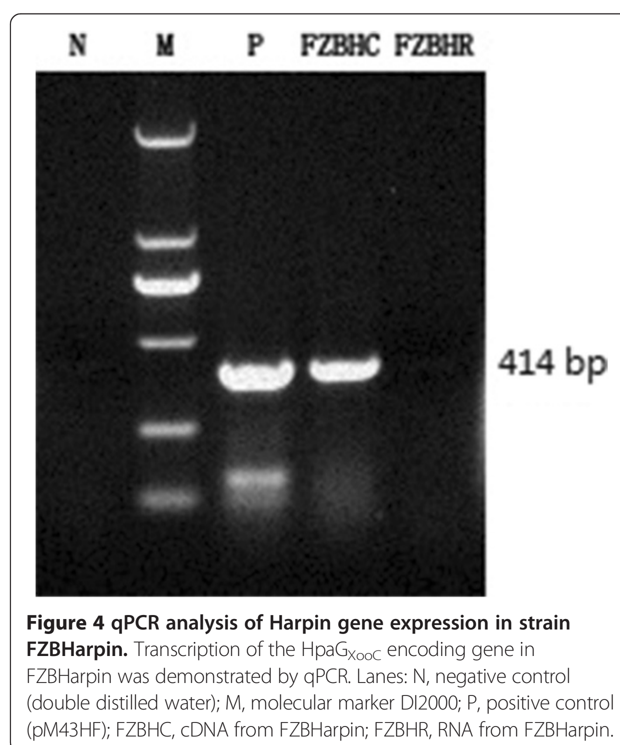

Figure 4 qPCR analysis of Harpin gene expression in strain FZBHarpin. Transcription of the $HpaG_{XooC}$ encoding gene in FZBHarpin was demonstrated by qPCR. Lanes: N, negative control (double distilled water); M, molecular marker DI2000; P, positive control (pM43HF); FZBHC, cDNA from FZBHarpin; FZBHR, RNA from FZBHarpin.

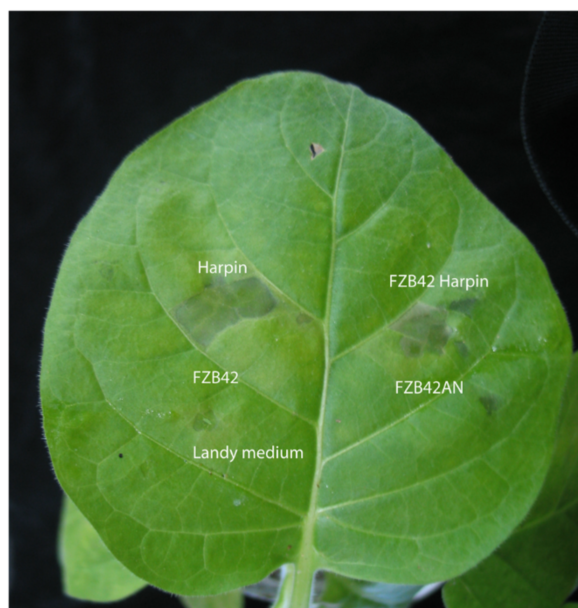

Figure 5 Hypersensitive response in tobacco elicited by FZBHarpin. The strains were cultivated in Landy medium, and the samples were taken after 72 h. One hundred microliters of the supernatant and of recombinant Hrp_{XooC} (15 μg · ml^{-1}) were applied onto tobacco leaf surfaces, and the hypersensitive response was checked after 24 h.

eviction of the resistance genes was observed in approximately one out of ten candidates analyzed and subsequently verified by PCR. Due to its thermo-sensitive origin of replication, the plasmid can be eliminated at non-permissive temperatures, and markerless deletion mutants can be obtained. Before applying the engineered FZB42Harpin under non-containment conditions, we have to perform marker removal by one of the methods described above.

Of course, marker removal is not the only precondition for improved acceptance of genetically engineered strains when released into the environment. As stated above, careful case studies demonstrating that no harmful effects caused by genetic engineered strains are urgently needed. In applying genetic engineered plant growth-promoting bacteria, we have to distinguish two different levels:

(1) Engineered strains without foreign genes but containing useful mutations in genes affecting the beneficial effect of the bacterium in terms of plant growth promotion and biocontrol of pathogens. Given that no resistance marker has been introduced, it might be unimportant whether the useful mutation has been introduced by a targeted allele exchange or has been evolved after applying a natural selection procedure. We believe that such strains will be accepted in the future when their improved action has been convincingly demonstrated.
(2) Engineered strains containing genes from bacteria. Such bacteria will be considered as 'recombinant', also when the donor bacteria occur in the same natural environment. This was the case in the example described here. Ironically, the *harpin* gene isolated from a pathogen bacterium was shown to act beneficial when cloned and expressed in FZB42. However, long-term environmental studies are necessary to demonstrate that such recombinant bacteria do not harm the environment by novel recombination events with other microorganisms occurring in the same environment.

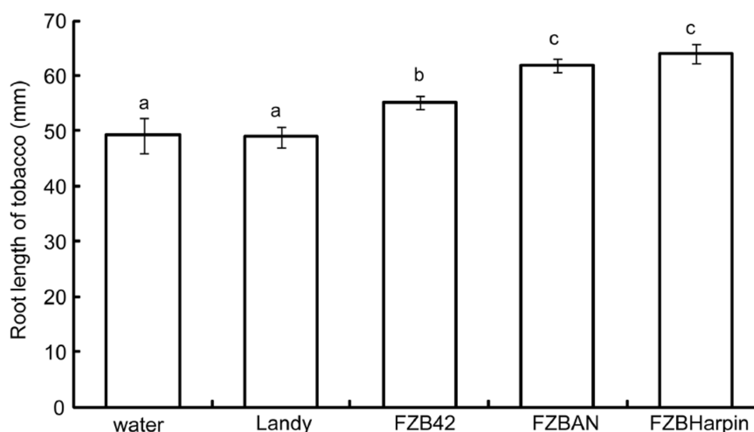

Figure 6 Promotion of root growth of tobacco plants grown in MS medium by FZB42Harpin. The lengths of the roots were determined as follows: control (water) 49.25 mm (±3.25), control (Landy medium) 48.95 mm (±1.92), FZB42 55.17 mm (±1.12), FZBAN (Δ*apr*, Δ*npr*) 61.79 mm (±1.23), and FZBHarpin (Δ*apr*, Δ*npr*, 2x hrp_{XooC}) 64.05 mm (±1.81).

Table 5 The biocontrol efficacy (rice bacterial blight) and plant growth promotion by FZB42, FZB42AN, and FZB42Harpin

Strain	Disease index (%)	Control efficacy (%)	Plant height (cm)
-	44.25 ± 2.82 a	-	71.75 ± 3.54 c
FZB42	36.57 ± 1.73 b	17.4	75.92 ± 1.88 b
FZBAN	28.97 ± 2.01 c	34.5	80.33 ± 1.53 a
FZBHarpin	21.26 ± 2.73 d	51.9	82.58 ± 0.80 a

Test was performed with tobacco plants. Groups designated as a, b, c are significantly different.

Conclusions

Biologicals prepared from beneficial microbes are useful and environmental-friendly tools for developing a sustainable and efficient agriculture. In this context, genomic analysis and genetic engineering of promising beneficial microbes are helpful for obtaining improved bioformulations. This strategy should enable us to save considerable amounts of agrochemicals, especially chemical fertilizers, and chemical pesticides.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JQQ carried out the molecular genetic studies and application experiments with FZB42Harpin and helped to draft the manuscript. HJW and RH carried out the molecular genetic studies and application experiments with FZB42Harpin. XWG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. RB designed the experiments and wrote the manunuscript. All authors read and approved the final manuscript.

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