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Agrobacterium tumefaciens Mediated Transformation of ChiV Gene to *Trichoderma harzianum*

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Abstract As a soil-borne filamentous fungus, *Trichoderma harzianum* exhibits biological control properties because it parasitizes a large variety of phytopathogenic fungi. In this study, the vectors pBI121 and pCAMBIA1301 and cloning vector pUC18 were used to successfully construct expression vector pCA-GChiV for filamentous fungi transformation mediated by *Agrobacterium tumefaciens*.The ChiV gene was successfully transferred into the biocontrol fungus *T. harzianum* with an efficiency of 90–110 transformants per 10^7 spores using *A. tumefaciens*-mediated transformation. Putative transformants were analyzed to test the transformation by the southern blot, and the expression of ChiV was detected by reverse transcription PCR. The transformants were co-cultured to assay antifungal activities with *Rhizoctonia solani*. The inhibition rates of the transformants and no ChiV gene transferred *T. harzianum* were 98.56% and 82.42%, respectively, on the fourth day.The results showed that the ChiV transformants had significantly higher inhibition activity.

Keywords *Trichoderma harzianum* · Biocontrol · *Agrobacterium tumefaciens*

Introduction

Chitinase plays a major role in the defensive strategies of plants against fungal pathogens because it decomposes chitin, the major component of most fungal cell walls, through

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catalyzing the hydrolysis of chitin, an unbranched polymer of β-1,4-*N*-acetylglucosamine [1]. Because of the ability to degrade fungal cell walls, chitinases were focused on manipulation and application of enhancing a plant's ability to resist fungal pathogens.

Therefore, chitinases are considered as having an important role in the defense response against phytopathogens. Chitinases are focal points for the control of phytopathogenic fungi [2]. Chitinases have been isolated from biocontrol fungi, such as *Trichoderma aureoviride* [3], *Trichoderma harzianum* [4], and *Chaetomium globosum* [5]. A chitinase gene was overexpressed from barley in *Escherichia coli* and the purified chitinase exerted broad-spectrum antifungal activity [6]. The chitinase gene chi36 from *T. harzianum* was expressed in yeast, and the recombinant chitinase chi36 produced by the yeast *Pichia pastoris* was shown to be active against different phytopathogens, confirming the importance of this endochitinase in the mycoparasitic activity of *Trichoderma* antagonistic strains [7]. The chi46 enzyme cloned from *C. globosum* in *Pichia pastoris* GS115 can efficiently degrade cell walls of the phytopathogenic *Rhizoctonia solani*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*, demonstrating that it may be involved in the biocontrol mechanism of *C. globosum* [5].

T. harzianum, an important plant biocontrol fungus with wide application, had important application value in the biocontrol of plant diseases, especially diseases infected through soil. *T. harzianum* can produce many kinds of antibiotics and ergosterols against plant fungal pathogens based on different mechanisms, such as the production of antifungal metabolites, competition for space and nutrients, and mycoparasitism. It was widely used in biocontrol of plant disease. Because the antimicrobial efficacy of *T. harzianum* was restricted, it was necessary to enhance the antimicrobial efficacy of *T. harzianum* in order to realize biocontrol function [8].

But until the most recent times, no chitinase gene with high-level expression has been transformed into *T. harzianum*. *Agrobacterium tumefaciens*-mediated transformation solved the technology problem,which has been further developed for using in yeast [9] and a number of filamentous fungi [9–11]. In the present work, we used the vectors pBI121 and pCAMBIA1301 and cloning vector pUC18 to successfully construct expression vector pCAGChiV for filamentous fungi transformation mediated by *A. tumefaciens*.The ChiV gene was first transformed into *T. harzianum* by *A. tumefaciens*-mediated transformation system, which was important for fungal development and biocontrol applications in the future.

Materials and Methods

Materials

T. harzianum was obtained from Hebei Agricultural University (China) and cultured in PD medium for 36 h at 26 °C [12]. *A. tumefaciens* AGL-1 was kindly provided by Prof. Chu Chengcai (Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences, China). Plant pathogen *R. solani*, pUC18, pBI121, and pCA1301 were obtained from the Department of Life Science and Engineering, Harbin Institute of Technology (China).

Isolation of Total RNA and mRNA

Approximately 400 mg of mycelium was ground to a fine powder under liquid nitrogen and transferred to a 50-mL Corning tube on ice. RNA was extracted from the mycelium of *T. harzianum* by Trizol reagent (Gibco). Messenger RNA (mRNA) was isolated by means of Oligotex mRNA Kits (Qiagen), according to the manufacturer's instructions. The quality of

the extracted total RNA was identified by a denaturing formaldehyde/agarose gel, and the concentration of mRNA was measured by spectroscopy.

Construction of cDNA Library

Approximately 8 μg of mRNA was used in a first-strand synthesis reaction catalyzed by the Superscript II-RT reverse transcriptase enzyme (Stratagene) with oligod(T)50 and *Xho*I adaptor as the primer. The second-strand complementary DNA (cDNA) was synthesized using the first-strand cDNA as the template. After being blunted to double cDNA, double-stranded cDNA (dscDNA) was linked with *Eco*RI adaptor and digested with *Xho*I, and the shorter dscDNAs (length<500 bp) were discarded by agarose electrophoresis. The size-fractionated cDNA was then purified with QiaEXII Gel Extraction Kit and ligated to the vector of pBluescript II SK+ (pBK) for overnight. One-microliter ligation sample was mixed up with competent *E.coli DH10a* (Invitrogen) and then electroporated with Micropulser (Bio-RAD) in 2.1 kV. The products were resuspensed with super optimal catabolite (SOC) medium and cultured in 37 °C for 45 min and then smeared in solid SOC medium plate in the presence of X-gal and isopropyl β-D-1-thiogalactopyranoside. Clone number and blue/white plaques ratio were calculated respectively.

DNA Sequencing and Analysis

Single plaque was randomly picked up and stored in YT buffer at 37 °C. The insert size of individual recombinant plaque was examined by specific PCR amplification by means of the T3 and T7 primers followed by 1.5% (*w*/*v*) agarose gel electrophoresis. Plasmid DNA was isolated from a 5-mL aliquot of the overnight culture using rapid plasmid isolation protocol and purified through QIAquick spin columns (Qiagen). Templates for DNA sequencing were prepared by specific PCR amplification of cDNA inserts directly by means of the T3 primers. Amplified inserts were purified with QIAquick spin columns (Qiagen) and sequenced with DyEnamic ET Terminator Sequencing Premix. Sequence data outputs were manually edited to remove vector and unreliable sequences. After DNA sequence analysis, translation, and alignment with related genes and proteins were carried out using the computer programs Lasergene (DNASTAR, Inc., Madison, WI, USA). The nucleotide sequence of cDNA was compared with the sequences in GenBank at the National Centre for Biotechnology Information (NIH) [13].

Bioinformatics Analysis of the ChiV Gene

The open reading frame and molecular mass were predicted by DNAMAN software. Theoretical isoelectric point of protein was calculated by Anthe5.0 program. The *T. harzianum* ChiV gene was deposited in the GenBank database, and the accession number was AY634683.

Construction of Binary Vector pCA-GC

pUC18 and pBI121 were digested with *Eco*RI and *Hin*dIII and ligated to form the plasmid pUC-GUS (containing NOS terminator and CaMV35S promoter) with the T4 DNA polymerase. The ChiV gene was amplified by means of the polymerase chain reaction. The resulting 1,194-bp fragment and pUC-GUS was digested with *Bam*HI and *Sac*I, and ligated to form the intermediate plasmid pUG-S. The larger fragment of pCA1301 was obtained by

*Eco*RI and *Hin*dIII and ligated with the little fragment of pUG-S digested by *Eco*RI and *Hin*dIII to form the last plasmid pCA-GC.

A. tumefaciens Cell Preparation

Agrobacterium strain AGL-1 was cultured by inoculating in 7 mL liquid LB (containing 50 μg/mL kanamycin and 50 μg/mL streptomycin) with a bit of frozen glycerol stock at 250 rpm, 29 °C overnight (16–20 h).The cells were diluted with the MM medium (containing 200 μmol/L acetosyringone, AS) to achieve an A_{660} of 0.15.The final volume should equal 20 mL. The *Agrobacterium* cells were grown for approximately 4 h at 29 °C, 250 rpm with final A_{660} from 0.6–0.8.

Spores of *T. harzianum* Preparation

Before *Agrobacterium* cells grew, *T. harzianum* spores from 1-week-old cultures were harvested with 5-mL sterile water on potato dextrose agar. The spore concentration was counted by a hemocytometer, and its suspension was diluted with MM medium to 10^5–10^6 spores/mL [14].

T. harzianum Transformation

One hundred microliter diluted spores were mixed with 100 μL *Agrobacterium* cells (A_{660}=0.6–0.8), and then the mixture could be spread evenly on MM medium (200 μmol/L AS) plates, incubating at 27 °C for 2 days. Co-cultivated after 2 days, M-100 medium (containing 200 μg/mL hygromycin and 300ug/mL Cefotaxime) was replated on the MM plates, and putative transformants would be visible after 5–7 days.

Southern Blot Analysis

Genomic DNA from *T. harzianum* was extracted from the Plant DNEeasy (Qiagen, Valencia, CA, USA). The sequences of hygromycin primers were as follows: hphR, 5′-TTCGATGTA GGAGGGCGTGGAT-3′ and hphF, 5′-CGCGTCTGCTGCTCCATACAAG-3′. Hygromycin PCR conditions were 94 °C for 4 min; 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1.5 min, 35 cycles; 72 °C for 10 min. The hygromycin of pCAMBIA1301 was used by making probes. Hybridization was implemented according to DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics Corporation, Germany) instruction manual.

Transformant Mitotic stability

The transformant was cultured on potato dextrose agar (PDA) without hygromycin B for determining the stability of the transformant. A mycelial plug could be taken from the edge of the culture and transferred onto fresh PDA by ten times repeated by colonization of each plate. The experiment by growing them on PDA containing hygromycin B showed resistance of these monoconidial cultures to hygromycin B.

RT-PCR Analysis

To identify differential expression of the defined chiV gene, mRNA was isolated by means of Oligotex mRNA Kits (Qiagen) according to the manufacturer's instruction.Thereafter, total cDNA was synthesized by RevertAid^{TM} M-MuLV reverse transcriptase (Promega) using oligo

$(dT)_{18}$ as a primer. The reverse transcription reaction was performed at 42 °C for 60 min and stopped by heating at 70 °C for 10 min. One microlitre aliquot of the reverse transcription reaction (25 μL) was taken to PCR amplification. The following primers were used for the identification of the genes: tublinR, 5′-TTTGACAGGGCAACCAAATCGGTGCCGCCT-3′; tublinF, 5′-CAGAGGATTGGCCGAAGATGAAGTTGTCGG-3′; chiVR, 5′-GCCGGATC CACCATGTCGGGAGACGGCTTTCGT-3′; and chiVF, 5′-GCCGAGCTCTCAATT GTTTGGGAATCCGTT-3′.

Assay of Fungal Transformants to Inhibition Activity

A block of transformant and pathogen colonies were cut respectively from the PDA plates on which transformant and pathogen were inoculated, and then inoculated on the two spots of PDA plate at 27 °C, and observed through the inhibition activity of fungal transformant to pathogen for 7 days. The inhibition activity test was as above. The assay was replicated three times in both cases.

Results

Sequence Analysis of ChiV Gene

A ChiV clone, containing the largest insert (1,360 bp), was screened from *T. harzianum* cDNA library after the sequence analysis. The ChiV gene consisted of the 1,360-bp sequence of nucleotides, including a 1,194-bp open reading frame, an ATG translation initiation codon, a TAA translation termination codon, and a poly (A) tail. This suggested that the cDNA contained the full-length coding sequence (Fig. 1). The full-length cDNA of 1,360 gene contained an uninterrupted open reading frame of 1,194 nucleotides, which encodes a 44-kDa polypeptide consisting of 397 amino acid residues. The theoretical isoelectric point was 5.18. The cDNA sequence of ChiV from *T. harzianum* had been accepted by GenBank, and the accession number was no. AY634683.

Construction of Binary Vector pCA-GC

The electrophoresis result of the plasmid pCA-GS, digested by *Bam*HI and *Sac*I (Fig. 2) showed that there were two bands at the position about 1,3031 and 1,194 bp, which represented the expressing vector pCA-C and ChiV gene, respectively. The results of double digestion showed that the recombination vector of pCA-GChiV was successfully constructed.

Screening of Fungal Transformants

After 5–7 days of plating on MM seclecting medium, the putative transformants were visible. When inoculating these transformants to a new plate containing 200 μg/mL hygromycin, they grew well even when hygromycin was up to 300 μg/mL. The results showed that the transformation efficiency was 90–110 transformants per 10^7 spores.

Molecular Biological Analysis of the Tranformants

DNA gel blots indicated that ChiV gene was integrated into the genome of *T. harzianum* as a single copy (Fig. 3). This showed that the ChiV transformation was successful.

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1     ACAAACATGTCGGGAGACGGCTTTCGTTCAGTCGCCTATTTCGTCAACTGGGCCATCTAT
1           M  S  G  D  G  F  R  S  V  A  Y  F  V  N  W  A  I  Y
61    GCTAGAAAGCATCGCCCCCAAGATCTCCCCGTTGATAAACTCACCCATATCCTCTATGCC
19     A  R  K  H  R  P  Q  D  L  P  V  D  K  L  T  H  I  L  Y  A
121   TTTGCCAATGTCCGCCAAGATAGCGGGGAAGTACACATGACGGATGGCTGGGCGGACACA
39     F  A  N  V  R  Q  D  S  G  E  V  H  M  T  D  G  W  A  D  T
181   GACATTCATTGGGAGGGTGACTCGTGGAATGACACGGGAAATAACATGTATGGCTGCCTC
59     D  I  H  W  E  G  D  S  W  N  D  T  G  N  N  M  Y  G  C  L
241   AAGCAGCTGAATCTTCTCAAGAAGCGCAATCGCAACCTCAAGGTTTTGCTGTCCATTGGC
79     K  Q  L  N  L  L  K  K  R  N  R  N  L  K  V  L  L  S  I  G
301   GGTTGGACGTACAGCGGCAACTTCAAGGGTCCGGCTAGTACTCAGCAGGGTCGTGAGACA
99     G  W  T  Y  S  G  N  F  K  G  P  A  S  T  Q  Q  G  R  E  T
361   TTCGCCAAATCCAGTCTCGAACTGCTCAAAAACTTGGGTTTCGATGGACTCGATATTGAT
119    F  A  K  S  S  L  E  L  L  K  N  L  G  F  D  G  L  D  I  D
421   TGGGAGTATCCTCAGAATGCGGACGAGGCTAGGAATTTCGTCGAGCTTCTCGCCACCGTG
139    W  E  Y  P  Q  N  A  D  E  A  R  N  F  V  E  L  L  A  T  V
481   CGCAGAGAGCTTGACGCCTACTCTGCCACCCTTCCAAACTACAGCCACTTTGAACTGACT
159    R  R  E  L  D  A  Y  S  A  T  L  P  N  Y  S  H  F  E  L  T
541   GTTGCTTGTCCCGCCGGAGCTACGCACTTCCAGAAGCTTGATGTCCCAGGAATGGACCGA
179    V  A  C  P  A  G  A  T  H  F  Q  K  L  D  V  P  G  M  D  R
601   TATCTTGATTTCTGGAACTTGATGGCTTACGACTATGCGGGGTCTTGGGACCAGACGAGC
199    Y  L  D  F  W  N  L  M  A  Y  D  Y  A  G  S  W  D  Q  T  S
661   GGCCATCAGGCGAACCTCCATCCGTCACTTGACAACCCTACGTCTACTCCCTTTTCTACC
219    G  H  Q  A  N  L  H  P  S  L  D  N  P  T  S  T  P  F  S  T
721   GATGCCGCCATCGACTTCTATACCAGGAGTGGTGTAGCACCCAGCAAGATTGTGCTCGGA
239    D  A  A  I  D  F  Y  T  R  S  G  V  A  P  S  K  I  V  L  G
781   ATGCCCATCTATGGTCGTGCATTTGAGAATACCGATGGCCCTGGCAGGCCGTACAATGGT
259    M  P  I  Y  G  R  A  F  E  N  T  D  G  P  G  R  P  Y  N  G
841   ATTGGTGAGGGGTCGTGGGAGAATGGCATCTTTGACTACAAGGTTTTCCCTCACCCAGGC
279    I  G  E  G  S  W  E  N  G  I  F  D  Y  K  V  F  P  H  P  G
901   TCTCAAGAAATTTGGGATCAAAAAACAGGCGCCAGCTACAGCTATAACCCCCAAACAAGG
299    S  Q  E  I  W  D  Q  K  T  G  A  S  Y  S  Y  N  P  Q  T  R
961   AAGCTTGTTTCCTTCGACCCCCCTCAAGCTAGTAGAGCCAAGGCTGGGTATATCAAGGAG
319    K  L  V  S  F  D  P  P  Q  A  S  R  A  K  A  G  Y  I  K  E
1021  TGGGGTTTTGGAGGTGGCATGTGGTGGGAGAGCAGCGGAGACAAGGAGGGCCCTGACAGC
339    W  G  F  G  G  G  M  W  W  E  S  S  G  D  K  E  G  P  D  S
1081  TTGATCGGTATTGTGGTGAATGAGTTTGGAGGCCCTGGGGCGTTGCAGAGGAAGGATAAC
359    L  I  G  I  V  V  N  E  F  G  G  P  G  A  L  Q  R  K  D  N
1141  TGCATCGACTATCCGCAGTCGAAATACGACAATTTGAAGAACGGATTCCCAAACAATTGA
379    C  I  D  Y  P  Q  S  K  Y  D  N  L  K  N  G  F  P  N  N  *
1201  GGAGAGGATCAAAATGCCCAATTCAAGCAGGTAGAACGAACGAGAACATGATATGACCCT
1261  GACATATAAATAAATCAGCGTAAATAGAAGATGAATAAAAAAGAATGATTTGAAAGTGAA
1321  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1 Nucleotide and deduced amino acid sequences of ChiV from *T. harzianum.* The cDNA sequence was translated from the first ATG codon in the open reading frame. The stop codom was TGA

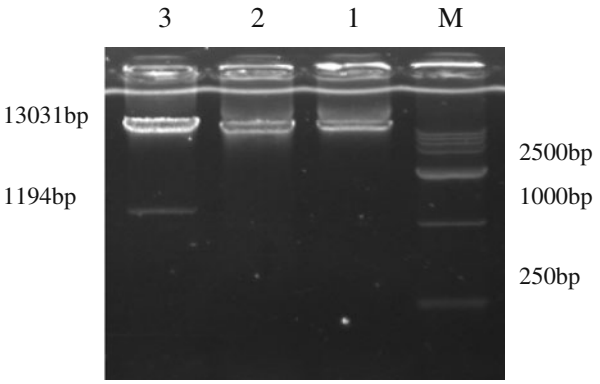

Fig. 2 The result of binary vector pCA-GC double enzyme digestion by *Bam*HI and *Sac*I. *M* marker DL15,000; *1–3* digested by *Bam*HI and *Sac*I

Mitotic Stability of the Transformants

The mitotic stability of the transformant was tested by growing them on PDA plates. After successively repeating through ten generations, we inoculated these transformants to PDA plates containing 200 μg/mL hygromycin. These transformants continued to grow up well, which showed that it was mitotically stable.

RT-PCR of the Transformants

Following RNA extraction from the control and transformant, RT-PCR was performed with tublin and chiV primers. The expression of the transformant chiV RNA was obviously higher than that of the wild strain (Fig. 4).

Inhibition Activity of the Transformant to Pathogen

The result showed that there was obvious difference between *T. harzianum* wild strain and its transformant on the inhibition activity to pathogen *R. solani*, and each of them strongly inhibited the growth of *R. solani* in normal circumstances. After 4–5 days dual culture with *R. solani, T. harzianum* gradually covered pathogen colony. The inhibit rates of wild strain and the transformant were 82.42% and 98.56%, respectively, on the fourth day (Table 1).

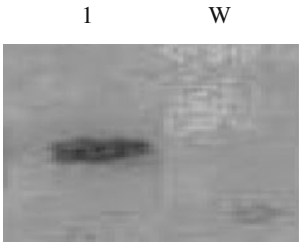

Fig. 3 Southern blotting of the ChiV transformant for hygromycin resistance gene. *1* ChiV transformant; *W* wild strain

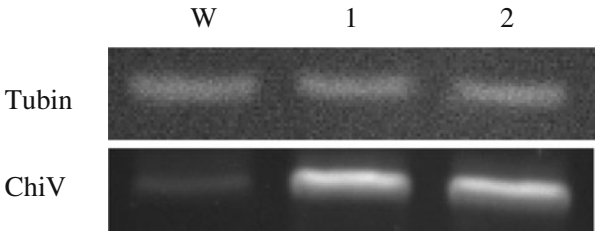

Fig. 4 RT-PCR analysis of *T. harzianum* transformants T-ChiV. *W* wild strain; *1* ChiV transformant cultured for 60 h; *2* ChiV transformant cultured for 72 h

Discussion

Trichoderma strains are inefficiently used against plant pathogens in terms of sensitivity to chemical fungicides, such as benzimidazole [15], a major component of chemical fungicides. In our laboratory, transformants resistant to benzimidazole of fungal origin have been successfully achieved by transferring a benzimidazole-resistant gene to *T. harzianum.* Transformants grow well even with a higher concentration of benzimidazole than the original concentration of benzimidazole in the medium [16, 17]. Therefore, it is an effective and practical way to apply *T. harzianum* as a biocontrol agent. To increase the chitinases activity in the process of biocontrol, *T. harzianum* was chosen as a model organism. Therefore, in this study, the ChiV gene was transformed to biocontrol fungus *T. harzianum* by *A. tumefaciens*-mediated transformation system. There was no obvious side effect on the growth of *T. harzianum* when ChiV gene was integrated into *T. harzianum*. The inhibit rate of the transformant was nearly 100% on the fourth day, which improved the ability of *T. harzianum* and shorten the time against plant pathogen *R. solani.* Constructing genetically engineered microbial strain of *T. harzianum* by ChiV gene recombinant technology increased the *T. harzianum* inhibition activity, which would be important for *T. harzianum* application in future.

In our laboratory, a *T. harzianum* mycelium cDNA library with insert sizes ranging from 800 to 4,000 bp was constructed, and the complexities of the library was over 1.2×10^6 PFU/ml. From this cDNA library, 4,128 clones were randomly sequenced and thereby identified 1,740 putative genes of *T. harzianum* [8]. We got some full-length biocontrol genes from the cDNA library. In this paper, the hygromycin resistance gene was a selected marker, and CaMV35S was high efficiency promoter, which could be used for genetic transformation of mycelial fungus. The construction of vector pCA-GChiV suggests a strategy for the enhancement of biocontrol gene expression in *T. harzianum*. The other biocontrol genes could be transformed in this method for improving the bioconrol ability.

In our privious study, we transformed the sod gene into the *T. harzianum* successfully [14]. In this study, the transformation system could transform into *T. harzianum* with an efficiency of 90–110 transformants/10^7 spores, which was higher than that of previous

Table 1 The inhibition growth (%) of *Rhizoctonia solani* by *T. harzianum*

Strain	The inhibition growth of *Rhizoctonia solani* (%)	
	4th day	7th day
Wild T88	82.42	100
T-ChiV	98.56	100

transformation of protoplast mediated by polyethylene glycol, overcoming the barriers confronted by conventional transformation protocols. A new technique was provided to the genetic modification of *T. harzianum* by *A. tumefaciens*-mediated transformation of *T. harzianum* [18, 19]. With its high transformation frequency and genetic stability of the transformants, the tranformation system mediated by *A tumefaciens* may prove to be beneficial for the filamentous fungi transformation and functional genomic research.

T. harzianum is a worldwide distributed fungus, whose potential in plant disease biological control is well-known. In the past research, chitinases have been expressed and characterized in the yeast and bacterial system. In this study, we transferred the chiV gene into the fungus *T. harzianum*, which improved the *T. harzianum* inhibition ability to the plant fungal pathogen *R. solani*. Therefore, expressing the ChiV gene highly in *T. harzianum* could help us develop a new way to tranform other biocontrol genes for applying in biology control and biotechnology engineering field in future.

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