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Construction of *Streptomyces lydicus* A01 transformant with the *chit33* gene from *Trichoderma harzianum* CECT2413 and its biocontrol effect on *Fusaria*

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Streptomyces lydicus A01 resists many plant pathogens (including *Fusarium* spp.) by producing the antifungal agent natamycin, which binds to the ergosterol of fungal cell membranes and inhibits the growth of pathogens. *Trichoderma harzianum* CECT2413 is a widely-distributed soil fungus that antagonizes several plant fungal pathogens (including *Fusarium* spp.) by producing chitinase and degrading chitin, a major component of the fungal cell wall. This study attempted to enhance the biocontrol effect of *S. lydicus* A01 on *Fusarium* spp. by transforming the chitinase gene of *Trichoderma*. Chitinase and natamycin could act synergistically on both the cell walls and cell membranes of pathogens. The 33-kD chitinase-encoding gene (*chit33*) was cloned and conjugal-transformed from *T. harzianum* CECT2413 to *S. lydicus* A01, and then confirmed via polymerase chain reaction (PCR) assays. Subsequent analyses using the 3,5-dinitrosalicylic acid (DNS) method and ultraviolet spectrophotometry showed that compared with its wild type strain (WT), the *S. lydicus* A01 conjugal transformant (CT) with *chit33* gene exhibited substantially higher chitinase activity and natamycin production. The resistance of *S. lydicus* A01-*chit33* CT and WT to four *Fusaria* in crops and vegetables was tested via the cup-plate method. Compared with the WT, the conjugal transformant of *S. lydicus* A01 with *chit33* gene from *T. harzianum* CECT2413 showed greatly increased biocontrol effect on fusarium disease. This study would be beneficial to the development of high-quality antifungal bio-agents for agricultural applications via the synergy between the previously non-existent and pre-existing functions achieved through heterogeneous gene transformation.

Streptomyces lydicus* A01, *chit33* conjugal transformant, wide-type strain, chitinase activity, natamycin production, *Fusarium

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Plant diseases, such as fusarium wilt, stalk rot, and root rot, cause substantial losses in the yield and quality of crops and vegetables such as cotton, peas, corn, and cabbages [1,2]. Fusarium diseases are mainly caused by *Fusarium* spp., which includes *Fusarium oxysporum* f.sp. *vasinfectum*, *Fusarium solani* f.sp. *pisi*, *Fusarium graminearum*, and *Fusarium oxysporum* f.sp. *conglutinans* [3–6]. The management of *Fusarium* remains difficult and the cultivation of disease-resistant varieties has achieved limited success. This result is partially due to the rapid mutation of pathogens. In addition, the application of chemicals, such as preplant soil fumigation with methyl bromide, is poisonous to humans [7]. Therefore, studies have focused on the prevention and biocontrol of fusarium diseases because of a need for high-quality antifungal agents.

Streptomyces lydicus A01 is a biocontrol agent that produces natamycin and is antagonistic to a variety of pathogens (including *Fusarium* spp.) [8]. Natamycin is an antibi-

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otic macrolide polyene commonly produced by *Streptomyces* spp., such as *Streptomyces natalensis* and *Streptomyces chattanoogensis*. Natamycin can specifically bind to the ergosterol in fungal cell membranes and then block fungal growth by inhibiting vacuole fusion [9,10]. Natamycin can resist *Fusarium* isolated from keratitis. As the only available anti-fungal medication approved by the American Food and Drug Administration, natamycin is not toxic to humans [11] and has long been used as a food additive [12,13].

Another important biocontrol agent, *Trichoderma* spp., has also been used on a number of crops and vegetables for the biocontrol of pathogens, including *Fusarium* spp. [14–16]. The cell wall-degrading enzymes produced by *Trichoderma harzianum*, such as chitinases (*chit33*), proteases (*prb1*), and β-glucanases (*bgn13.1*), have been described as antagonistic components against *F. oxysporum* [17]. Compared to its wide-type strain, the *T. harzianum* Rifai TM transformants that overexpress the *chit36* gene can more efficiently inhibit *F. oxysporum* [18]. Chitinase can hydrolyze the chitin, a β-1, 4-linked polymer of *N*-acetyl glucosamine present in most fungi (including *Fusarium* spp.) as a major cell wall component [19].

Natamycin and chitinase, produced by *Streptomyces* and *Trichoderma*, respectively, are common biocontrol agents used in the biocontrol of fungal diseases. To date, only a few chitinases have been identified in *Streptomyces* spp., such as a 49-kD chitinase from *Streptomyces griseus* HUT 6037, with no chitinase detected in *S. lydicus* A01 [20]. Previous studies have attempted to enhance the antifungal activity of *Streptomyces* against pathogens mainly by enhancing their production of antibiotics production using ultraviolet mutation, chemical mutagenesis, or intraspecific protoplast fusion [21,22]. No study has examined the combined biocontrol effects of natamycin and chitinase on specific plant diseases related to *Fusarium* spp. thus far. Combining the high chitinase expression of *Trichoderma* with the substantial natamycin production of *Streptomyces* via transformation is a worthwhile goal. In this study, an engineering strain that can express both chitinase and natamycin was constructed via the conjugal transformation of the *chit33* gene from *T. harzianum* CECT2413 to *S. lydicus* A01. Chitinase activity and natamycin production were tested for both *S. lydicus* A01-*chit33* CT and wild-type (WT) via the 3,5-dinitrosalicylic acid method (DNS) and ultraviolet spectrophotometry. The associated anti-*Fusaria* activities of *S. lydicus* A01-*chit33* CT and WT were examined via the cup-plate method. The results of this study could provide insights into the development of high-quality anti-fungal bio-agents through heterogeneous gene transformation.

1 Materials and methods

1.1 Plasmids and strains

The pIB139 and pHZ1272 vectors were provided by BAI LinQuan (School of Life Science and Biotechnology, Shanghai Jiao Tong University). The pIB139 vector, which carries an apramycin resistance cassette and the promoter region of the erythromycin resistance gene (ermE* promoter), could express exogenetic protein constitutively by inserting the cassette into the *Streptomyces* genome. The DEV intermediate vector was purchased from Generay Biotech Co., Ltd. (China).

The cloning host, *Escherichia coli* strain DH5α, was purchased from Takara (Japan) and the donor for conjugal transformation, ET12567 (pUZ8002), was provided by Linquan Bai. The recipient strain, *S. lydicus* A01, and the pathogens, *F. graminearum*, *F. oxysporum* Schl. f.sp. *conglutinans*, *F. solani* f.sp. *pisi*, and *F. oxysporum* f.sp. *vasinfectum*, were provided by Liu Weicheng (Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences).

1.2 Synthesis and amplification of the sequences

The *chit33* gene of *T. harzianum* CECT2413 [23] was synthesized according to the partial codons of *Streptomyces coelicolor* for high expression in *S. lydicus* A01 (Generay, China). In addition, the DNA sequence of the melC1 signal peptide of *Streptomyces* was synthesized [24]. The terminator sequence of *Streptomyces* was polymerase chain reaction (PCR)-amplified from the pHZ1272 vector with *Ter*-F1 and *Ter*-R2 primers (Table 1). The PCR conditions were as follows: 95°C for 10 min, followed by 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and finally, 72°C for 10 min.

The DNA sequence of the melC1 signal peptide was 5′-CGGGGTCGTCAACCCAACGCACCCCAGGAGGTCCCGCATGCCGGAACTCACCCGTCGTCGCGCGCTCGGCGC-3′. The sequence of the terminator was 5′-AAAGTTTTG-TC-GTCTTTCCAGACGTTAGTAAATGAATTTTCTGTATGAGGTTTTGCTAAACAACTTTCAACAGTTTCAGCGGAGTGAGAATAGAAAGGAACAACTAAAGGAATTGCGAATAATAATTTTTTCACGTTGAAAATCTCCAAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATTTCTTAAACAGCTTGATACCGATAGTTGCGCCGACAATGACAACAACCATCGCCCACGCATAACCGATATATTCGGTCGCTGAGGCTTGCAGGGAGTCAAAGGCCGCTTTTGCGGGATCTCGTCGAAGGCGGCGGGGGCGCCGGACGCGGCCGGGTTCCCGGG-3′.

Table 1 The primers used in this study

Primer name	Primer sequence (5′→3′)
Ter-F1	AAAGATATCCCCAAGCTTAAAGTTTTGTCGTCT
Ter-R2	CCGGAATTCCCCGGGAACCCGGCCGCG
Apr-F1	CGTCAACTCCAAGCAGAACA
Apr-R2	CTCGAAGTCGAAGTCGAAGC
Chit33-F1	GCTCATCGGTCAGCTTCTCA
Chit33-R2	TCGCATTCTTCGCATCCC

1.3 Gene cloning and conjugal transformation

The construction procedure of the positive pIB139 vector was plotted into four cycles using Winplas 2.7 (Figure 1). The positive pIB139 vector was identified via digestion with *Nde* I and *Eco*R I, transformed to ET12567 (pUZ8002) for conjugal transformation to *S. lydicus* A01, and then analyzed using PCR assays. A blank pIB139 vector was transformed to verify whether it can affect chitinase activity and natamycin production without the *chit33* gene. The conjugal transformation was performed according to Kieser et al. [25]. The genomes were extracted using a G+ bacteria genomic DNA kit (Zoman, China). Two pairs of primers were used to screen the putative ex-conjugants, that is, *S. lydicus* A01-*chit33* CT via PCR assays, including *S. lydicus* A01 conjugal transformant with a blank pIB139 vector (*S. lydicus* A01-pIB139 CT). *Chit33*-F1 and *Chit33*-R2 were used to amplify part of the *chit33* gene. *Apr*-F1 and *Apr*-R2 were used to amplify part of the apramycin-resistant gene in pIB139 (Table 1). This amplification was conducted under the following conditions: 95°C for 10 min; 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min.

1.4 Chitinase activity assay

The chitinase activity assay was performed with the help of a chitinase-inductive broth consisting of the seed broth (0.5% glucose, 0.5% tryptone, 0.5% yeast extract, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$) and the fermentation broth (1% chitin powder, 0.4% NH_4NO_3, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$). Briefly, the mycelia of *S. lydicus* A01-*chit33* CT and WT (including *S. lydicus* A01-pIB139 CT) were inoculated to the seed broth, which was then incubated on a 180 r min^{-1} rotary shaker at 28°C for 2 d. The precipitation of the seeds (100 μL) was subsequently added to the fermentation broth, and the supernatant was obtained via centrifugation every two days from Days 2 to 14. Chitinase activity was determined using the DNS method [26]. The *N*-acetyl-*D*-glucosamine (NAG) standard curve was prepared according to Reissig et al. [27]. One unit (U) of chitinase activity was defined as the amount of enzyme required to release 1 μg of *N*-acetyl-β-glucosamine per hour.

Chitin-hydrolysis capability (diameter of the hydrolysis zone) was examined for the supernatants of the 10-d old fermentation broth of *S. lydicus* A01-*chit33* CT and WT. Briefly, 200 μL of the supernatant was inoculated to each Oxford cup on chitin-containing agar plates (0.3% colloidal chitin, 0.4% NH_4NO_3, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, and 2% agar powder). The plates were incubated at 37°C in the dark, and the diameter of the chitin-hydrolysis zone was measured after 12 h. Each measurement was performed at least in triplicate.

1.5 Natamycin production assay

Natamycin (Aladdin, China) solutions in methanol with different concentrations were measured at A_{303} using ultraviolet spectrophotometry and a standard curve was prepared. Natamycin production was then determined according to the standard curve by measuring the absorbance of the fermentation supernatant dilution (1:10, v/v, diluted in the methanol) of 2-d-, 6-d-, and 10-d-old *S. lydicus* A01-*chit33* CT and WT (including *S. lydicus* A01-pIB139 CT) in the chitinase-inductive broth at 303 nm [8]. Each measurement was performed at least in triplicate.

1.6 Inhibition test of *S. lydicus* for biocontrol of *Fusaria*

Exactly 200 μL of the fermentation supernatants of 10-d old *S. lydicus* A01-*chit33* CT and WT in chitinase-inductive broth were added to two separate Oxford cups on the plates containing 25 mL of potato dextrose agar (PDA) supplemented with *Fusaria* spore suspension (final concentration 10^6 cfu mL^{-1}). The plates were incubated at 28°C for 2 d,

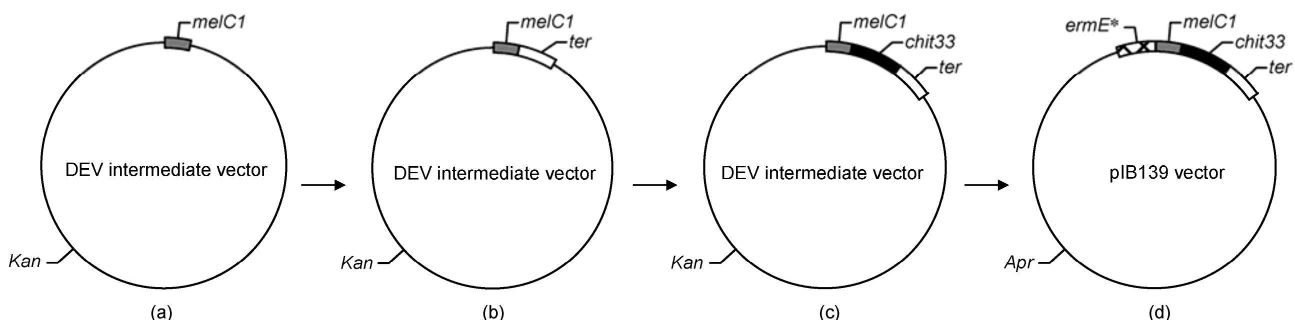

Figure 1 Cloning diagram of the *chit33* gene. (a) The DNA sequence encoding the melC1 signal peptide inserted into the DEV intermediate vector after digestion with *Nde* I and *Bam*H I; (b) Terminator sequence ligated to the signal sequence in the vector after digestion with *Eco*R V and *Eco*R I; (c) Synthesized *chit33* gene inserted between the signal sequence and the terminator after digestion with *Bam*H I and *Hin*d III; (d) Fragment containing the DNA sequence of melC1 signal peptide, *chit33* gene, and terminator gene excised from the DEV vector and inserted after the ermE* promoter in the pIB139 vector after digestion with *Nde* I and *Eco*R I.

and the diameter of the *Fusaria* inhibition zone was measured for *S. lydicus* A01-*chit33* CT and WT. Each measurement was performed at least in triplicate.

2 Results

2.1 Conjugal transformation of the *chit33* gene into *S. lydicus* A01

The sequence similarity (grey area) and difference (white area) of the *chit33* gene before and after optimization were 81.7% and 18.3%, respectively (Figure 2). The positive pIB139 vector containing *chit33* was identified via digestion with *Nde* I and *Eco*R I. The pIB139 vector sequence was 5922 bp in length, and those of the melC1 signal peptide, *chit33* gene, and the terminator were 1438 bp. The results were in agreement with the predetermination values (Figure 3). The *S. lydicus* A01-*chit33* CT was identified via PCR assay (including *S. lydicus* A01-pIB139 CT). The theoretical lengths of the apramycin and *chit33* genes amplicons in the pIB139 vector were 728 and 436 bp, respectively. The practical amplified results corresponded to the theoretical values. The pIB139 vector containing the *chit33* gene (including the blank pIB139 vector) was inserted into the *S. lydicus* A01 genome, and engineering strains were successfully constructed (Figure 4).

2.2 Chitinase activity of *S. lydicus* A01

Compared with its WT, *S. lydicus* A01-*chit33* CT displayed higher chitinase activity from Days 4 to 14 during fermentation, particularly from Days 10 to 14 during the stationary phase. On Day 10, the chitinase activity of *S. lydicus* A01-*chit33* CT was 452.664 ± 30.537 U mL^{-1}, which was 7.3 times that of the WT (61.921 ± 14.927 U mL^{-1}). The chitinase activity of *S. lydicus* A01-pIB139 CT was 65.656 ± 13.364 U mL^{-1} on Day 10, comparable to that of WT (Figure 5(a)). The heterogenous gene *chit33* from *T. harzianum* CECT2413 was highly expressed, and the insertion of the pIB139 vector into the genome had nearly no impact on the chitinase expression of *S. lydicus* A01.

Further comparison of the chitin-hydrolysis haloes were only made between the fermentation supernatants of the *S. lydicus* A01-*chit33* CT and WT on Day 10. The diameter of the hydrolysis zone produced by the fermentation supernatant of *S. lydicus* A01-*chit33* CT averaged 1.3 ± 0.1 cm, substantially larger than that of *S. lydicus* A01 WT (average 0.4 ± 0.1 cm, Figure 5(b)). The results of the chitin-hydrolysis haloe analysis were consistent with those of the chitinase activity analysis via DNS method.

2.3 Natamycin production of *S. lydicus* A01

The natamycin production of *S. lydicus* A01-*chit33* CT and WT (including *S. lydicus* A01-pIB139 CT) were examined at three time points. The natamycin production of *S. lydicus* A01-*chit33* CT on Days 2, 6, and 10 were 1.677 ± 0.706, 18.182 ± 1.161, and 40.576 ± 2.817 μg mL^{-1}, respectively, and those of WT were 1.424 ± 0.517, 2.758 ± 0.911, and 5.970 ± 1.318 μg mL^{-1}, respectively. The natamycin production of *S. lydicus* A01-*chit33* CT was 6.8 times that of the WT on Day 10, both proportional to chitinase activity in *S. lydicus* A01-*chit33* CT and WT. By contrast, the natamycin production of *S. lydicus* A01-pIB139 CT on Days 2, 6, and 10 were 1.364 ± 0.833, 2.697 ± 0.964 and 5.818 ± 1.289 μg mL^{-1}, respectively, with no substantial differences from those of the WT (Figure 6).

Thus, the pIB139 vector had nearly no effect on the

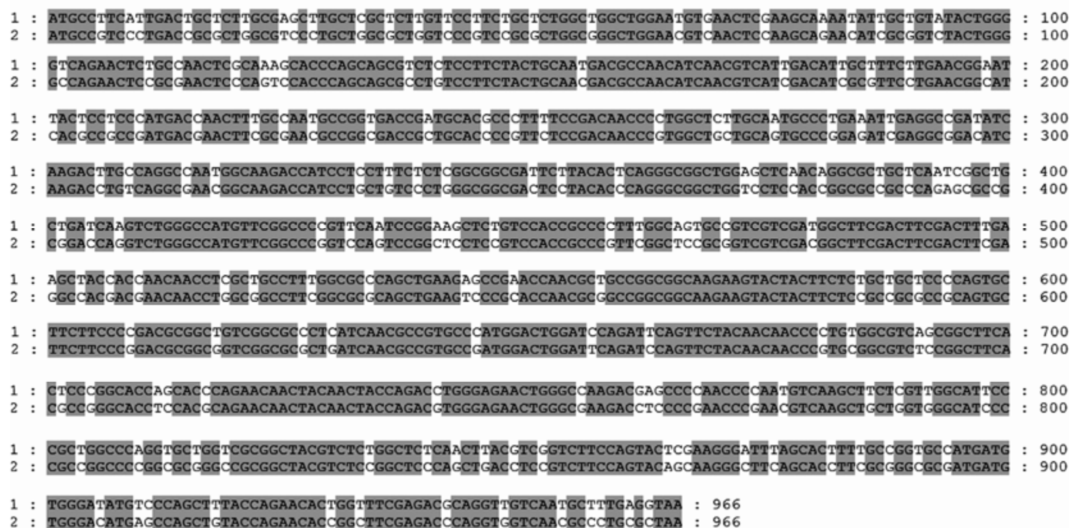

Figure 2 BLAST analysis of *chit33* gene from *T. harzianum* CECT2413 and optimization according to the codon preferred by *S. coelicolor*. 1, Original *chit33* gene from *T. harzianum* CECT2413; 2, The optimized *chit33*.

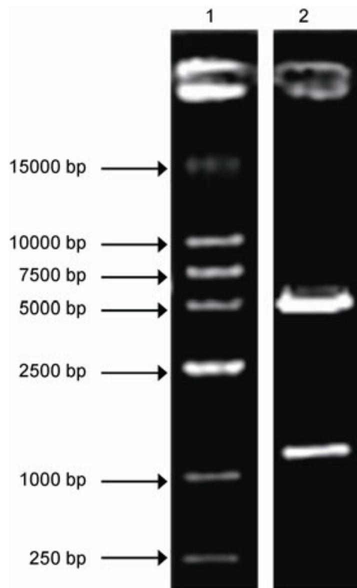

Figure 3 Gel image of positive pIB139 vector via double-digestion. 1, Marker DL15000 (TaKaRa, Japan); 2, positive pIB139 after double-digestion.

natamycin production of *S. lydicus* A01. In fact, natamycin production increased substantially rather than decreased after the transformation of the *chit33* gene.

2.4 Biocontrol effect of *S. lydicus* A01 against *Fusaria*

The diameters of the *Fusaria* inhibition zone produced by the 10-d-old fermentation supernatant of *S. lydicus* A01-*chit33* CT and WT were 1.7 – 1.8 cm and 0.35 – 0.8 cm, respectively. The biocontrol effect of the fermentation supernatant produced by *S. lydicus* A01-*chit33* CT on *Fusaria* was at least twice that of the WT (Figure 7). These results indicate that the inhibition effect of *S. lydicus* A01-*chit33* CT on *Fusaria* was substantially greater than that of *S. lydicus* A01 WT.

3 Discussion

This study attempted to improve the biocontrol effect of *S. lydicus* A01 against *Fusarium* spp. via the conjugal transformation of *chit33* from *T. harzianum* CECT2413. The *in vitro* tests showed that chitinase expression was substantially increased via the optimization of codon, the use of signal peptide, and the deployment of the ermE* strong promoter. The associated natamycin production of *S. lydicus* A01-*chit33* CT was also substantially improved. These results suggest the possibility of synergizing the natamycin production of *S. lydicus* A01 and the chitinase activity of *Trichoderma* through *chit33* gene transformation. In addition, a blank pIB139 vector was transformed to *S. lydicus* A01 in order to determine whether it affected the chitinase expression and natamycin production without the *chit33* gene. Because *S. lydicus* A01-pIB139 CT and WT are little different in terms of chitinase activity and natamycin production, we inferred that the pIB139 vector had no impact. Therefore, the *S. lydicus* A01-pIB139 CT was no longer analyzed and only *S. lydicus* A01-*chit33* CT and WT were subsequently discussed.

In this study, the induction broth used for the chitinase

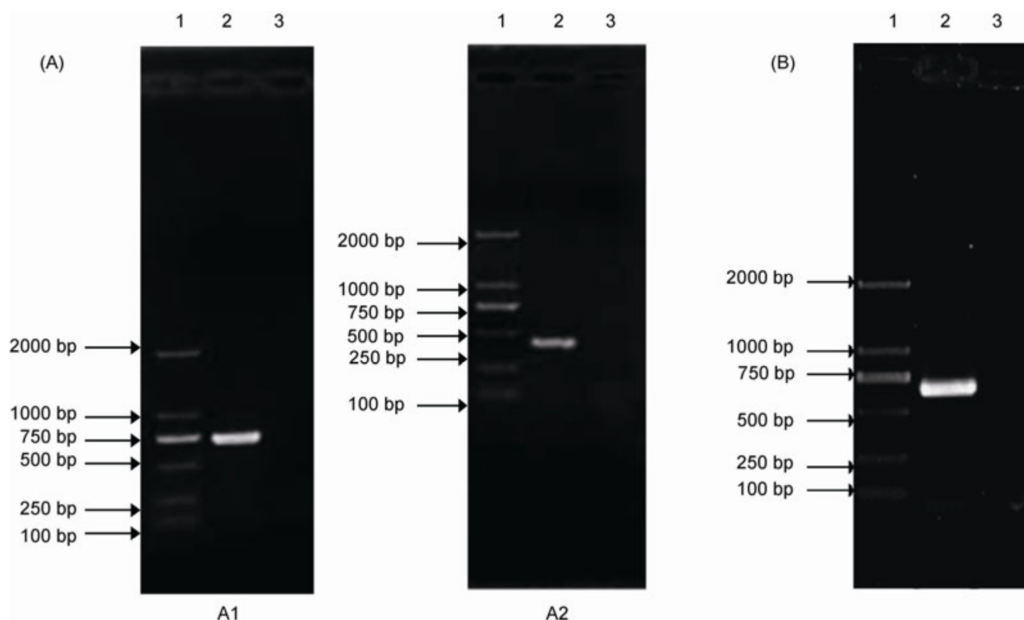

Figure 4 Gel image of *S. lydicus* A01-*chit33* CT and *S. lydicus* A01-pIB139 CT via PCR assay. A1, Apramycin-resistant gene amplification: 1, Marker DL2000 (TaKaRa, Japan); A2, *S. lydicus* A01-*chit33* CT; 3, *S. lydicus* A01 WT. A2, *Chit33* gene amplification: 1, marker DL2000; 2, *S. lydicus* A01-*chit33* CT; 3, *S. lydicus* A01 WT. (B) Apramycin-resistant gene amplification: 1, marker DL2000; 2, *S. lydicus* A01-pIB139 CT; 3, *S. lydicus* A01 WT.

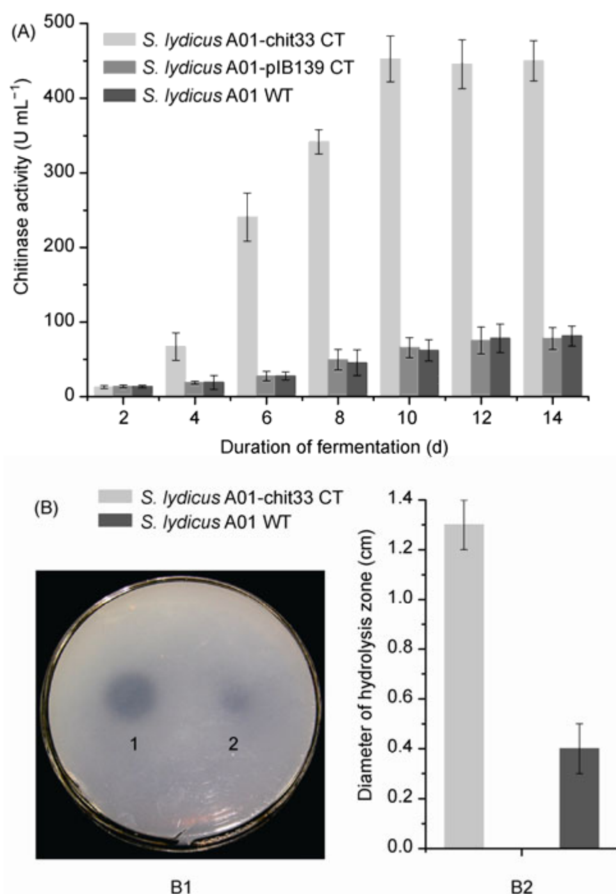

Figure 5 Chitinase activity analysis. (A) Chitinase activities of *S. lydicus* A01-*chit33* CT, *S. lydicus* A01-pIB139 CT, and WT; B1, Diameters of chitin-hydrolysis-haloes produced by *S. lydicus* A01-*chit33* CT and WT. 1, *S. lydicus* A01-*chit33* CT; and 2, *S. lydicus* A01 WT; and B2, Statistic analysis of chitin-hydrolysis-haloes.

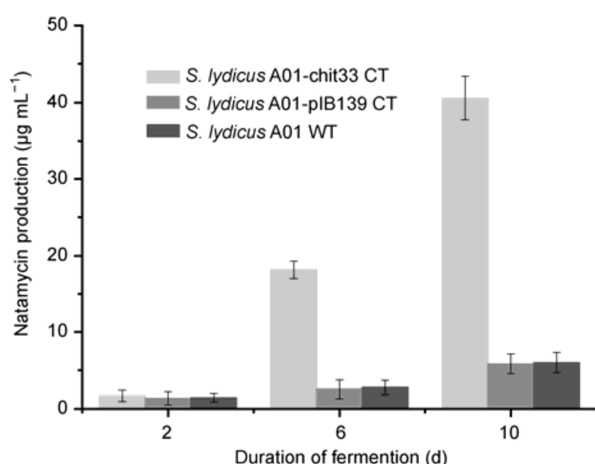

Figure 6 Natamycin productions of *S. lydicus* A01-*chit33* CT, *S. lydicus* A01-pIB139 CT and WT.

activity assay contained chitin powder as the sole carbon source. This chitin powder stimulated the chitinase expression of *S. lydicus* A01-*chit33* CT. Although most fungi or bacteria use colloidal chitin as the carbon source because they may not efficiently use chitin powder (e.g., *Trichoderma harzianum* TUBF 781, *Trichoderma koningi* with *chit42* of *Metarhizium anisopliae*, and *Streptomyces* sp. M-20), *S. lydicus* A01-*chit33* CT metabolized chitin powder with high efficiency [28–30]. Therefore, this engineering strain can be used in plant disease control and industrial chitin degradation.

The chitinase activity of both *S. lydicus* A01-*chit33* CT and WT increased over time during the 10-d fermentation, and the associated natamycin production increased correspondingly. Owing to the high over-expression of *chit33*, the chitinase activity of *S. lydicus* A01-*chit33* CT was substantially higher, consistent with the increase in natamycin production (Figures 5(a) and 6). Therefore, we hypothesize that chitinase degraded chitin into glucose, which was further absorbed and utilized by *S. lydicus* A01 for cell growth and natamycin production. Because the *S. lydicus* A01-*chit33* CT expressed much more chitinase than its WT, the former could grow faster and thus produce more natamycin.

The anti-*Fusaria* effect of the *S. lydicus* A01-*chit33* CT was at least twice that of the WT via the synergy of natamycin and chitinase (Figure 7). However, natamycin and chitinase production level barely met the needs of agriculture. The associated culture broth needs to be improved by supplementing yeast extract powder and/or tryptone as carbon and nitrogen sources, except for the chitin powder and ammonium nitrate, to improve the chitinase activity and natamycin production of *S. lydicus* A01-*chit33* CT. The broth pH, incubation temperature, and rotation speed should also be optimized [28,31].

Because of their strong secretory system, *Streptomyces* can express several exogenous genes from fungi, animals, and humans, such as interferon-α1 and interleukin-2 in humans expressed in *Streptomyces lividens* [32,33]. However, further study is needed to examine whether multiple functional genes can be transformed into *S. lydicus* A01 as novel high-quality biocontrol agents.

It is of importance to ensure that *S. lydicus* A01-*chit33* CT has no environmental pollution or disruption effects based on environmental toxicity tests. Our future studies include *in vitro* experiments with vegetables and crops to solve more practical problems.

4 Conclusions

In this study, a conjugal transformant of *S. lydicus* A01 was constructed using the *chit33* gene from *T. harzianum* CECT2413. As a result, the chitinase was highly expressed in *S. lydicus* A01-*chit33* CT, and the associated natamycin production was substantially increased. Our results show that the fermentation supernatant produced by *S. lydicus* A01-*chit33* CT had a much stronger biocontrol effect on *Fusaria* in crops and vegetables in the PDA plate, compared with the supernatant produced by the WT. To our knowledge,

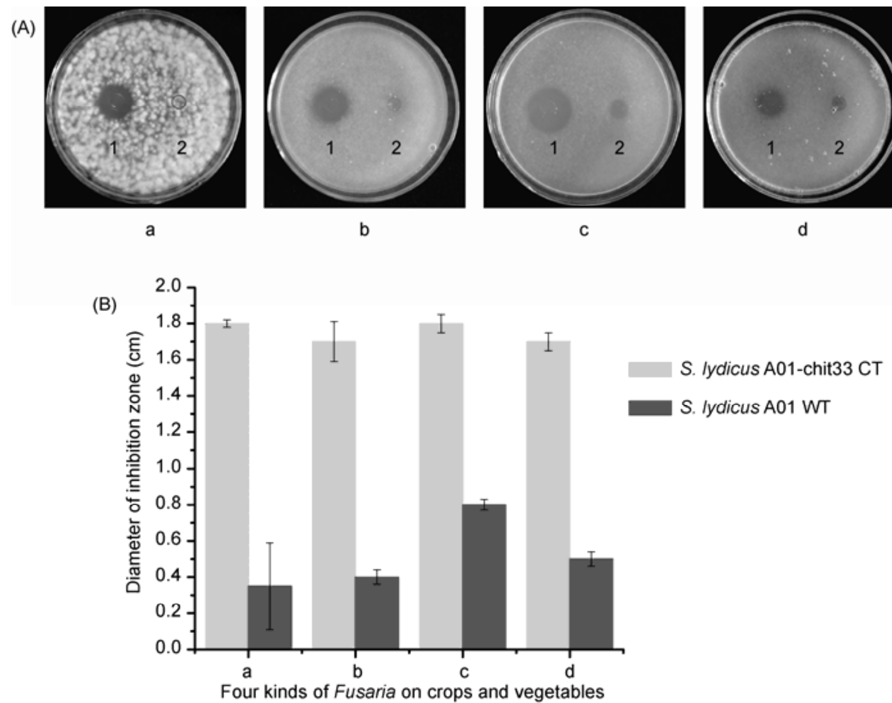

Figure 7 Control effects of *S. lydicus* A01-*chit33* CT and WT against *Fusaria*. (A) Photos showing the control effects of *S. lydicus* A01-*chit33* CT and WT against 4 kinds of *Fusarium*; a, *Fusarium graminearum*; b, *Fusarium oxysporum* Schl. f.sp. *conglutinans*; c, *Fusarium solani* f.sp. *pisi*; and d, *Fusarium oxysporum* f.sp. *vasinfectum*; 1, inhibition zones produced by *S. lydicus* A01-*chit33* CT; and 2, inhibition zones produced by *S. lydicus* A01 WT. (B) Statistic analysis of the inhibition zones.

this study is first to present evidence for the improvement of the biocontrol effect of *Streptomyces* on *Fusaria* via the synergy of natamycin and chitinase effects. These findings could significantly affect the research and development of high-quality biocontrol agents through genetic transformation.

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