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Construction of a *Streptomyces lydicus* A01 Transformant with a *chit42* Gene from *Trichoderma harzianum* P1 and Evaluation of Its Biocontrol Activity against *Botrytis cinerea*

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***Streptomyces lydicus* A01 and *Trichoderma harzianum* P1 are potential biocontrol agents of fungal diseases in plants. *S. lydicus* A01 produces natamycin to bind the ergosterol of the fungal cell membrane and inhibits the growth of *Botrytis cinerea*. *T. harzianum* P1, on the other hand, features high chitinase activity and decomposes the chitin in the cell wall of *B. cinerea*. To obtain the synergistic biocontrol effects of chitinase and natamycin on *Botrytis cinerea*, this study transformed the *chit42* gene from *T. harzianum* P1 to *S. lydicus* A01. The conjugal transformant (CT) of *S. lydicus* A01 with the *chit42* gene was detected using polymerase chain reaction (PCR). Associated chitinase activity and natamycin production were examined using the 3, 5-dinitrosalicylic acid (DNS) method and ultraviolet spectrophotometry, respectively. The *S. lydicus* A01-*chit42* CT showed substantially higher chitinase activity and natamycin production than its wild type strain (WT). Consequently, the biocontrol effects of *S. lydicus* A01-*chit42* CT on *B. cinerea*, including inhibition to spore germination and mycelial growth, were highly improved compared with those of the WT. Our research indicates that the biocontrol effect of *Streptomyces* can be highly improved by transforming the exogenous resistance gene, i.e. *chit42* from *Trichoderma*, which not only enhances the production of antibiotics, but also provides a supplementary function by degrading the cell walls of the pathogens.**

***Keywords*:** *Streptomyces lydicus* A01, *chit42* from *Trichoderma harzianum* P1, *Botrytis cinerea*, chitinase activity, natamycin production

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Introduction

On a global scale, gray mold diseases caused by *Botrytis cinerea* are widespread, leading to heavy economic losses in vegetable and fruit production (Alfonso *et al*., 2000). *Streptomyces* species are known to produce a variety of antibiotics (e.g., validamycin and natamycin) with excellent activities against pathogenic fungi in the agricultural and food industries (Ishikawa *et al*., 2005; Pintado *et al*., 2010). Natamycin is a polyene macrolide antibiotic with broad-spectrum antifungal activity against plant pathogens, including *B. cinerea*. It prevents the growth of fungi by specifically binding to ergosterol without permeabilizing the cell membrane and the ß-ring of ergosterol plays an important role in the natamycin-ergosterol interaction (te Welscher *et al*., 2008, 2010). *S. lydicus* strain A01, an anti-fungal organism isolated from a vegetable soil in Beijing, China, has been proven to be stable for producing natamycin and controlling *B. cinerea*-related plant diseases (Lu *et al*., 2008).

Trichoderma is another biocontrol agent widely used for controlling pathogenic fungi, including *B. cinerea*. One of its main antifungal mechanisms is hyperparasitism along with secretion of multiple extra-cellular enzymes, such as chitinase, protease and glucanase, which are major components involved in the decomposition of the cell walls of pathogens (Lorito *et al*., 1994, 1996; Elad and Kapat, 1999; Liu *et al*., 2010). Chitinase, which could degrade the chitin of fungal cell wall to N-acetyl-D-glucosamine (GlcNAc), including chitin oligomers, plays a crucial role in hyperparasitism (Elad *et al*., 1983; Cortes *et al*., 1998; Monte, 2010). In addition, disruption of the *chit42* gene in *Trichoderma harzianum* P1 has been reported to reduce its inhibitory effect on *B. cinerea* (Woo *et al*., 1999). Compared with the wild type, transgenic lemons with the *chit42* gene from *T. harzianum* have shown substantially higher control of *B. cinerea* (Distefano *et al*., 2008). Since chitinase has not been detected in *S. lydicus* A01, combining the chitinase-related characteristics of *Trichoderma* and natamycin-related characteristics of *Streptomyces* via transformation may be a worthwhile undertaking. Theoretically, the transformant can simultaneously achieve high chitinase activity and yield natamycin production, further inhibiting *B. cinerea* via synergistic effects on the cell wall and cell membrane.

In the present study, the *chit42* gene was transformed from *T. harzianum* P1 into *S. lydicus* A01 wild-type strain (WT), and the *S. lydicus* A01-*chit42* conjugal transformant (CT) was detected using polymerase chain reaction (PCR). Chitinase activity and natamycin production were tested using the 3,

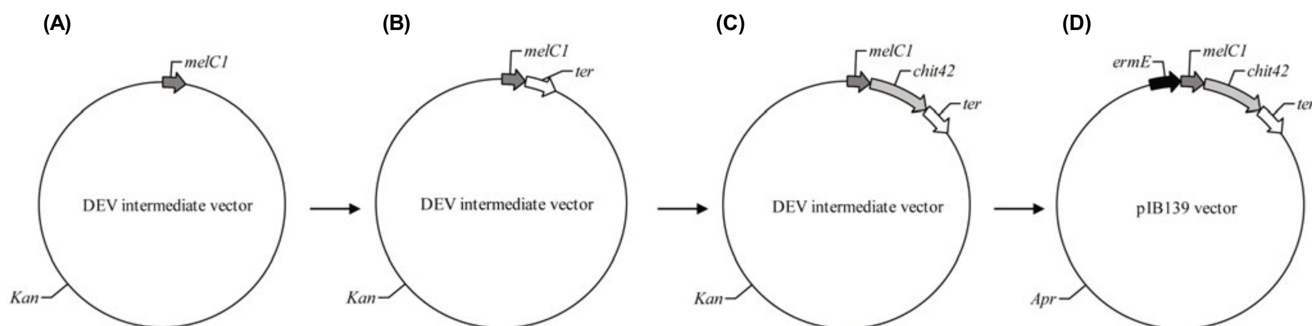

Fig. 1. Cloning diagram of the *chit42* gene from *T. harzianum* P1. (A) The DNA sequence of *melC1* signal peptide linked to DEV intermediate vector after digestion with *Nde*I and *Bam*HI. (B) The terminator sequence linked after digestion with *Eco*RV and *Eco*RI. (C) The *chit42* gene linked after digestion with *Bam*HI and *Hind*III. (D) Upon digestion with *Nde*I and *Eco*RI, the fragment containing the DNA sequence of *melC1* signal peptide, *chit42* gene and terminator sequence inserted after the *ermE** promoter in the pIB139 vector.

5-dinitrosalicylic acid (DNS) method and ultraviolet spectrophotometry, respectively. The *in vitro* control effects were investigated and results provided evidences of substantially improved inhibitory effects of *S. lydicus* A01-*chit42* CT on *B. cinerea* in terms of spore germination and mycelial growth, compared with the WT.

Materials and Methods

Plasmids and strains

The pIB139 vector, which carries an apramycin resistance cassette and an ermE* promoter, was kindly provided by Linquan Bai (School of Life Science and Biotechnology, Shanghai Jiao Tong University, China). The pIB139 vector could insert into the genome of *S. lydicus* A01 by conjugal transformation for constitutive protein expression. The DEV vector was used as an intermediate vector during the construction of the positive pIB139 plasmid with *chit42*.

Escherichia coli strains DH5a and ET12567 (pUZ8002) were used as the cloning host and donor for conjugal transformation, respectively. A wild-type strain of natamycin-producing *S. lydicus* A01, kindly provided by Weicheng Liu (Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, China), was used as the recipient. A strain of *B. cinerea* isolated from sick tomato leaves, also kindly provided by Weicheng Liu, was used as the target pathogenic fungus for examination of the biocontrol effects of *S. lydicus* A01-*chit42* CT and WT.

Cloning of the *chit42* gene

The *chit42* gene of *T. harzianum* P1 (Mach *et al*., 1999) was synthesized (Generay, China) according to the codon preference of *Streptomyces coelicolor* for high expression in *S. lydicus* A01. The *melC1* signal peptide sequence of *Streptomyces* was synthesized in order to guide chitinase42 secretion into the extracellular environment (Hong *et al*., 2003). The terminator from the pHZ1272 vector of *Streptomyces* was used to stop the transcription process of the *chit42* gene.

The construction procedure of positive pIB139 vector with *chit42* was plotted by Winplas 2.7 software and is shown in Fig. 1. The positive pIB139 vector was verified by double-enzyme digestion and then transformed into *E. coli* ET12567 (pUZ8002) for conjugal transformation.

Conjugal transformation

For conjugal transformation, 200 μl of *E. coli* ET12567 (pUZ 8002) with the positive pIB139 vector were added into 20 ml of LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl), supplemented with apramycin (final concentration 30 μg/ml), kanamycin (final concentration 50 μg/ml), and chloramphenicol (final concentration 25 μg/ml), and then grown to an OD600 of 0.5–0.6. The cells were harvested by centrifugation at 4,000 r/min for 5 min, washed twice with LB broth, and then suspended in 2 ml of LB broth. Fifty microliters of freshly cultured *S. lydicus* A01 spores were suspended in 500 μl of 2*YT broth (1.6% tryptone, 1% yeast extract, and 0.5% NaCl), incubated at 50°C for 10 min for germination, and then cooled to room temperature. Five hundred milliliters of *E. coli* cell suspension (donor) were added to 500 μl of A01 spore suspension (recipient), and the mixture was spread on MS plates (2% D-mannitol, 2% soybean-cake powder, and 2% agar) supplemented with $MgCl_2$ (final concentration 10 mmol/L). After incubation at 28°C for 16 h, the conjugation plates were overlaid with 1 ml of ddH_2O containing 500 μg of nalidixic acid and 1 mg of apramycin, and then incubated at 28°C for another 2 d. The ex-*S. lydicus* A01-*chit42* CTs were transferred to TSBY broth (1% pancreatic soy peptone, 0.5% yeast extract, and 10.3% sucrose) with nalidixic acid (final concentration 25 μg/ml) and apramycin (final concentration 30 μg/ml), and then incubated on a 180 r/min rotary shaker at 28°C for 4 d. The mycelia were harvested for extraction of genomic DNAs, which were used as templates for PCR identification (Kieser *et al*., 2000). The partial sequence of the *chit42* gene was amplified using the sense primer 5′-GTACGCCGACTAC CAGAAGC-3′ and the anti-sense primer 5′-TGTTGTAC GGGGAGGAGTTC-3′. The *apramycin*-resistance gene in pIB139 was amplified using the sense primer 5′-GCTCAT CGGTCAGCTTCCA-3′ and the anti-sense primer 5′-TCG CATTCTTCGCATCCC-3′. PCR amplification was conducted under the following conditions: 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and 72°C for 10 min. The quality of PCR products

were checked by 1% agarose gel electrophoresis.

Chitinase activity assay

Chitinase activity was determined using the DNS reagent (Harman *et al*., 1993). A GlcNAc standard curve was prepared according to Reissig *et al*. (1955). One unit (U) of the chitinase activity was defined as the amount of enzyme required to release 1 μg of GlcNAc per h.

For chitinase production, two broths were used, i.e., 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, mycelia of *S. lydicus* A01-*chit42* CT and WT were inoculated into the seed broth and incubated on a 180 r/min rotary shaker at 28°C for 2 d. One hundred microliters of seed precipitation were added to the fermentation broth and incubated on a 180 r/min rotary shaker at 28°C for 14 d. The fermentation supernatant was obtained by filtering the mycelia through a 0.45 μm sterile membrane every 2 d. Absorbances were measured at 540 nm using the UV-1800 ultraviolet spectrophotometer (Mapada, China), and the results were used to calculate the chitinase activities. Each treatment was performed in triplicate.

The hydrolysis of chitinase was examined in a 9 cm diameter Petri dish containing 25 ml of chitinase production medium (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). Two Oxford cups were placed on the plate and individually loaded with 200 μl of 10 d old filtered fermentation supernatant of *S. lydicus* A01 CT or WT. The plates were incubated at 37°C for 24 h, after which the diameters of the chitin hydrolysis zones were measured. Each treatment was performed in triplicate.

Natamycin production assay

The ten day old filtered fermentation supernatants of *S. lydicus* A01-*chit42* CT and WT were diluted 1:10 in methanol (v/v), respectively, and absorbances were measured at the three ultraviolet absorption wavelengths of natamycin, i.e., 303 nm, 290 nm, and 318 nm (Lu *et al*., 2008). The natamycin productions of *S. lydicus* A01-*chit42* CT and WT were calculated according to the standard curve prepared using natamycin (Aladdin, Japan) solutions in methanol at different concentrations. Each treatment was performed in triplicate.

Assay of antagonistic effect on spore germination of *B. cinerea*

Two Oxford cups were placed on an agar plate containing 25 ml of potato dextrose agar (PDA) with *B. cinerea* spore suspension (final concentration 10^6 CFU/ml), and individually loaded with 200 μl of 10 d old filtered fermentation supernatants of *S. lydicus* A01-*chit42* CT and WT, respectively. The plates were incubated at 28°C for 2 d, after which the diameters of the *B. cinerea*-inhibition zones were measured. Each measurement was performed in triplicate.

Twenty milliliters of the fermentation supernatant of *S. lydicus* A01-*chit42* CT or WT, containing 10% potato dextrose (PD) broth, were added to a conical flask and another conical flask containing 20 ml of un-inoculated fermentation broth with 10% PD broth as a control (CK). Spore pellets of *B. cinerea* were added at a density of 10^6 CFU/ml and the flasks were incubated on a 120 r/min rotary shaker at 25°C. Spore germination was examined under the light microscope after each 12 h. Each treatment was performed in triplicate.

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         10        20        30        40        50        60        70        80        90       100       110       120       130
A : ATGTTGGGCTTCCTCGGAAAATCCGTGGCCCTGCTTGCTGCGCTGCAGGCCACTCTCATTTCTGCATCTCCTGTAACTGCAAACGACGTCTCTGTTGAGAAGAGAGCCAGTGGATACGCAAACGCCGTCTACTTC
B : ...C..........G..C..G.....C........G..G.............G..G..C..C..CAGC..C..C..C..G........G..C..C......C.C..GTCC..C.....C.....G.........

       140       150       160       170       180       190       200       210       220       230       240       250       260       270
A : ACCAACTGGGGTATTTACGGCCGCAACTTCCAGCCTCAGAACCTGGTCGCGTCGGACATCACTCATGTCATCTACTCGTTCATGAACTTCCAAGCAGACGGCACTGTCGTCTCTGGAGATGCCTACGCCGATTAT
B : ..G........C..C...................C...........G...AGC........G..C..G......AGC..............G..C........C..G...AGC..C..C..G........C..C

         280       290       300       310       320       330       340       350       360       370       380       390       400
A : CAGAAGCACTATGACGACGATTCTTGGAACGACGTCGGTAACAATGCGTACGGCTGTGTGAAGCAGCTGTTCAAGCTGAAGAAGGCCAACCGCAACTTGAAGGTTATGCTTTCCATCGGTGGCTGGACCTGGTCC
B : ..........C........C..C..............C.....C...........C.....................................C.......G.....G........C............AG.

       410       420       430       440       450       460       470       480       490       500       510       520       530       540
A : ACCAACTTTCCTTCTGCAGCAAGCACCGATGCCAACCGCAAGAACTTTGCCAAGACTGCCATCACCTTCATGAAGGACTGGGGTTTCGATGGTATTGACGTCGATTGGGAGTACCCCGCCGATGATACCCAGGCC
B : ........C..G..C..G..CTC...G..C.................C........C..G.......................C.....C..C..C....G..C...........G..G..C..C..G.....G

         550       560       570       580       590       600       610       620       630       640       650       660       670
A : ACCAACATGGTTCTTCTGCTCAAGGAGATCCGATCTCAGCTAGATGCCTATGCTGCGCAATACGCTCCGGGCTACCACTTCCTTCTTTCCATTGCTGCCCCCGCTGGCCCAGAGCACTACTCTTTCCTGCACATG
B : ...........C..G.....G...........C..C.....G..C.....C..G.....G.....G.................G..GAG...C..G.....G..G.....G...........C...........

       680       690       700       710       720       730       740       750       760       770       780       790       800       810
A : TCCGACCTTGGCCAAGTTCTCGACTATGTCAACCTCATGGCCTACGACTATGCTGGTTCTTGGAGCAGCTACTCCGGACACGATGCCAACTTGTTTGCCAACCCGTCCAACCCCAACTCTTCACCATACAACACC
B : ........G.....G..G..G.....C........G.....G........C..G..C..C...TC.TC.........C.....C..G...C....C..G..............G.....C..C..G.........

         820       830       840       850       860       870       880       890       900       910       920       930       940
A : GATCAAGCTATCAAGGACTATATCAAGGGAGGTGTTCCCGCAAGCAAGATCGTTCTTGGCATGCCCATCTACGGACGAGCTTTTGAGAGCACCGGTGGCATTGGCCAGACCTACAGTGGAATTGGATCTGGAAGC
B : ..C..G..G...........C........C..C..C..G..G...........C..G........G.........C..C..G..C...TC......C.....C............TCC..C..C..CAGC..CTC.

       950       960       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
A : TGGGAGAACGGTATTTGGGACTACAAGGTTCTTCCCAAGGCCGGCGCCACAGTCCAGTATGACTCTGTCGCACAGGCATACTACAGCTATGACCCCAGCAGCAAGGAGCTCATCTCTTTCGATACCCCTGACATG
B : ...........C..C...............G..G..G.....G.....G..C.........C.....C.....G.....C......TC...C.....GTC.TC.........G...AGC.....C..G..G......

         1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200      1210
A : ATCAACACCAAGGTCTCTTACCTCAAGAACCTCGGCCTGGGAGGCAGCATGTTCTGGGAAGCTTCTGCTGACAAGACTGGCTCTGACTCCTTGATCGGAACAAGCCACAGAGCTTTGGGAAGCCTAGACTCCACT
B : ........G......AGC.....G........G........C...TC............G..CAGC..C........G...AGC......C.......C..GTC....C.C..CC....CTC...G........C

      1220      1230      1240      1250      1260      1270
A : CAGAACTTGCTGAGCTACCCCAACTCCCAGTATGATAACATCCGAAGCGGTCTCAACTAG
B : ......C.....TC......G...AG......C..C.........CTC...C..G.....A
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Fig. 2. **BLAST analysis of the *chit42* genes from *T. harzianum* P1 and optimized according to the codon preference of *S. coelicolor*.** (A) The *chit42* gene from *T. harzianum* P1. (B) The optimized *chit42* gene.

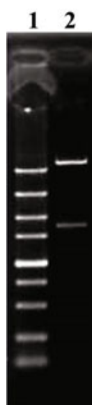

Fig. 3. Gel image of the positive pIB139-*chit42* vector identified by double-enzyme digestion. Lanes: 1, Marker DL5000 (TaKaRa, Japan); 2, the positive pIB139-*chit42* vector.

Assay of antagonistic effect on mycelial growth of *B. cinerea*

Ten day old fermentation supernatants of *S. lydicus* A01-*chit42* CT and WT were collected and filtered through the 0.45 μm sterile membranes. Twenty-five milliliters of PDA medium, containing 35% fermentation supernatant of *S. lydicus* A01-*chit42* CT or WT, were added into a 9 cm diameter Petri dish, while another petri dish contained 25 ml of PDA medium with 35% supernatant of un-inoculated fermentation broth as control (CK). The PDA plate covered in *B. cinerea* mycelia was punched with a 7 mm-diameter punch, then one little block was placed in the middle of each upper dish and incubated at 25°C for 4 d. The diameters of inhibition-zones were measured every day. The edges of the colonies after a 3 d incubation were cut with a sterile scalpel and observed under the DM2500 M light microscope (Leica, Germany). Each treatment was performed in triplicate.

Results

Cloning and conjugal transformation of the *chit42* gene

Sequences of the *chit42* gene before and after optimization were blasted by Genedoc software (Fig. 2). Figure 2A shows the original sequence of *chit42* from *T. harzianum* P1, while Fig. 2B shows the sequence of *chit42* optimized according to the codon preference of *S. coelicolor* (optimized area shown only).

The positive pIB139 vector with *chit42* was identified via double-enzyme digestion. The sequence of the pIB139 vector was 5,922 bp in length and the fragment containing *melc1*, *chit42*, and the terminator was 1,746 bp in length (Fig. 3).

The positive pIB139 vector containing the over-expression cassette of *chit42* was integrally inserted into the genome of *S. lydicus* A01 (Fig. 4A). PCR detection of the *S. lydicus* A01-*chit42* CT showed that the amplified fragments of the *apramycin*-resistant gene from the pIB139 vector and the *chit42* gene were 728 bp and 551 bp, respectively (Figs. 4B and 4C).

Chitinase activity of *S. lydicus* A01

During the incubation period, *S. lydicus* A01-*chit42* CT displayed higher chitinase activities than *S. lydicus* A01 WT on days 4–14, with a plateau on days 10–14. As for the 10 d old culture, the chitinase activity of *S. lydicus* A01-*chit42* CT was 435.020±25.010 U/ml, 6.9 times that of the WT (63.338±9.928 U/ml) (Fig. 5A). The diameters of the chitin hydrolysis zones produced by *S. lydicus* A01-*chit42* CT and WT were 1.20±0.10 cm and 0.43±0.06 cm, respectively (Figs. 5B and 5C). These findings confirmed that the *chit42* gene was highly expressed in *S. lydicus* A01.

Natamycin production of *S. lydicus* A01

The absorbances of *S. lydicus* A01-*chit42* CT at 290 nm, 318 nm, and 303 nm were consistently 5.6 to 6.3 times that of *S. lydicus* A01 WT (Table 1). As the absorbance of natamycin reached its maximum at 303 nm, subsequent tests only focused on the data obtained at 303 nm. According to the standard curve, the natamycin production of 10 d old *S. lydicus* A01-*chit42* CT was 36.030±0.864 μg/ml, 6.2 times that of the WT (5.849±0.946 μg/ml) (Fig. 6). The results suggested that the natamycin production of *S. lydicus* A01-*chit42* CT was greatly increased compared with the WT.

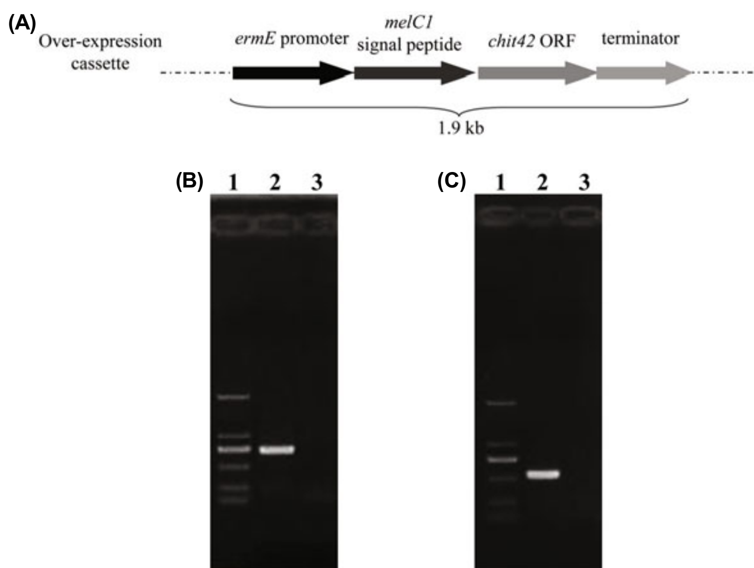

Fig. 4. Gel images of *S. lydicus* A01-*chit42* CT and WT detected by PCR. (A) The over-expression cassette of *chit42*. (B) Results of *apramycin*-resistant gene amplifications. Lanes: 1, Marker DL2000 (TaKaRa, Japan); 2, *S. lydicus* A01-*chit42* CT; 3, WT. (C) Results of *chit42* gene amplifications. Lanes: 1, Marker DL2000; 2, *S. lydicus* A01- *chit42* CT; 3, WT.

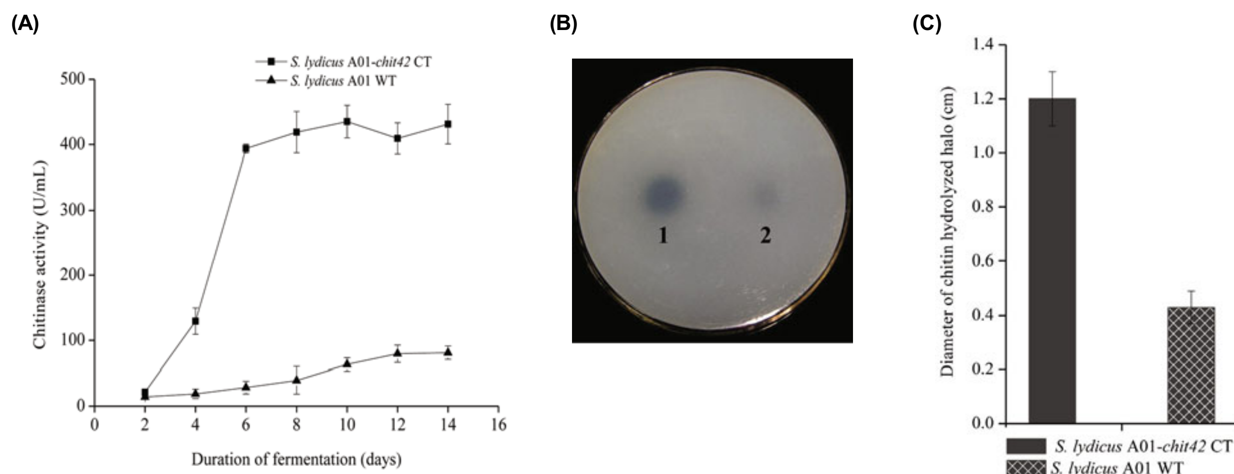

Fig. 5. Chitinase activity analysis. (A) Chitinase activities of *S. lydicus* A01-*chit42* CT and WT. (B) Chitin-hydrolyzed haloes produced by fermentation supernatants of *S. lydicus* A01-*chit42* CT and WT. Lanes: 1, *S. lydicus* A01-*chit42* CT; 2, WT. (C) Statistic analysis of the chitin-hydrolysis-haloes.

Control effect of *S. lydicus* A01 on spore germination of *B. cinerea*

The diameters of *B. cinerea*-inhibition zones produced by the fermentation supernatants of 10 d old *S. lydicus* A01-*chit42* CT and WT averaged 2.17±0.06 cm and 0.73±0.06 cm, respectively. The control effect of the fermentation supernatant of *S. lydicus* A01-*chit42* CT on *B. cinerea* spore germination was about three times that of the WT (Figs. 7A and 7B).

In addition, the *B. cinerea* spores treated with fermentation supernatant of *S. lydicus* A01-*chit42* CT showed no germination after incubation for 12 h, whereas those in the CK had a germination rate of almost 100% with long mycelia. The germination rate of *B. cinerea* spores treated with *S. lydicus* A01 WT was partial and the length of their mycelia was much shorter than those subjected to CK treatment. After incubation for 36 h, pretty small amounts of *B. cinerea* spores treated with *S. lydicus* A01-*chit42* CT barely started to germinate and produced very short mycelia. *B. cinerea* spores in *S. lydicus* A01 WT and CK treatments had a germination rate of 100% with very long mycelia, whereas the length of mycelia in the former were still shorter than those in the latter (Fig. 7C).

These indicated that, comparing with the WT, the inhibition to spore germination of *B. cinerea* by *S. lydicus* A01-*chit42* CT was substantially improved.

Control effect of *S. lydicus* A01 on mycelial growth of *B. cinerea*

The colony diameters of *B. cinerea* cultured on PDA plates containing fermentation supernatants of *S. lydicus* A01-*chit42* CT, the WT and CK, respectively, were measured on days 1–4, as the column diagram indicated in Fig. 8A. In addition, the pictures of *B. cinerea* colonies after a 3 d incubation were displayed intuitively in Fig. 8B. The colony diameters of *B. cinerea* on PDA plates containing the fermentation supernatant of *S. lydicus* A01-*chit42* CT was only 1.4 cm±0.1 cm, whereas that on plates containing the fermentation supernatant of *S. lydicus* A01 WT and control broth with no *S. lydicus* (CK) were 5.5 cm±0.2 cm and 6.7 cm±0.2 cm, respectively (Fig. 8B). Under the light microscope, in the CK treatment, the *B. cinerea* mycelia were found to be sparse, free and barely branching. In comparison, the *B. cinerea* mycelia treated with the fermentation supernatant of *S. lydicus* A01-*chit42* CT were dense, intricate and highly branching. The density and branch frequency of *B. cinerea* mycelia treated with *S. lydicus* A01 WT were slightly higher than CK (Fig. 8C). These observations indicated that the inhibition to mycelial growth of *B. cinerea* by *S. lydicus* A01-*chit42* CT was substantially strengthened as compared with the WT.

Table 1. Absorbances of fermentation supernatants of *S. lydicus* A01-*chit42* CT and WT at three ultraviolet absorption wavelengths of natamycin

	290 nm	303 nm	318 nm
S. lydicus A01-*chit42* CT	0.352±0.008	0.404±0.010	0.367±0.003
S. lydicus A01 WT	0.056±0.014	0.072±0.010	0.061±0.003

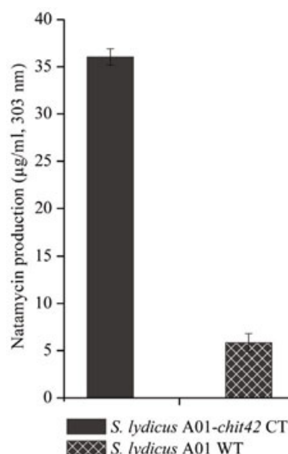

Fig. 6. Natamycin productions of *S. lydicus* A01-*chit42* CT and WT.

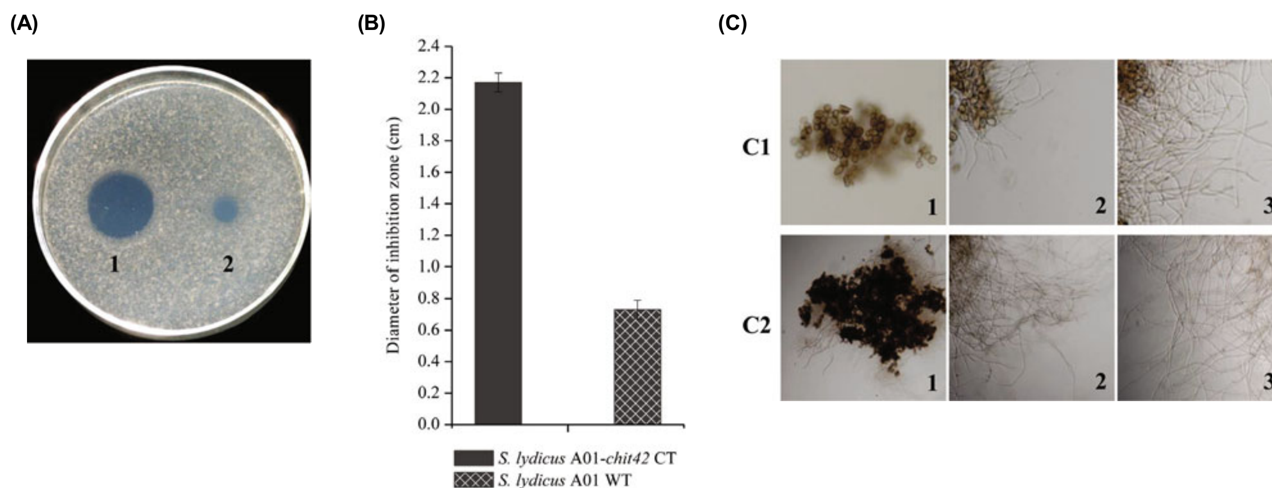

Fig. 7. Control effects of *S. lydicus* A01-*chit42* CT and WT on spore germination of *B. cinerea*. (A) Inhibition zones produced by *S. lydicus* A01-*chit42* CT and WT. Lanes: 1, *S. lydicus* A01-*chit42* CT; 2, WT. (B) Statistic analysis of the inhibition zones. (C) Micrographs showing the spore germination of *B. cinerea* treated with fermentation supernatants of *S. lydicus* A01-*chit42* CT, WT and CK; (C1) 12 h. Lanes: 1, *S. lydicus* A01-*chit42* CT; 2, WT and 3, CK; (C2) 36 h. Lanes: 1, *S. lydicus* A01-*chit42* CT; 2, WT and 3, CK.

Discussion

The control effect of *Trichoderma* on *B. cinerea* has previously been enhanced via over-expression of chitinase genes. Enhancing the production of chitinase42 in *T. harzianum* CECT2413 improves its inhibition to *B. cinerea* (Limón *et al*., 2004). Over-expression of the *chit36* gene in *T. harzianum* TM has shown substantially inhibitory effects on the germination of *B. cinerea* conidia (Viterbo *et al*., 2001). Several studies have attempted to improve the antifungal activity of *Streptomyces* against pathogens (including *B. cinerea*) through improvements in the antibiotic production via ultraviolet mutation, chemical mutagenesis, and/or intraspecific protoplast fusion (Chen *et al*., 2007; Wang *et al*., 2009). To date, however, no studies have reported the over-expression of chitinase genes from *Trichoderma* in *Streptomyces* to enhance the biocontrol of *B. cinerea* via the synergistic effects of chitinase and antibiotics.

In the present study, a biocontrol agent was constructed through transforming the *chit42* gene from *T. harzianum* P1 into *S. lydicus* A01. Laboratory tests showed that the chitinase production of *S. lydicus* A01-*chit42* CT was substantially improved to about 6.9 times that of *S. lydicus* A01 WT (Fig. 5A). The former also showed substantially high natamycin production, yielding 6.2 times more natamycin than the latter (Fig. 6). We speculated the reason was that,

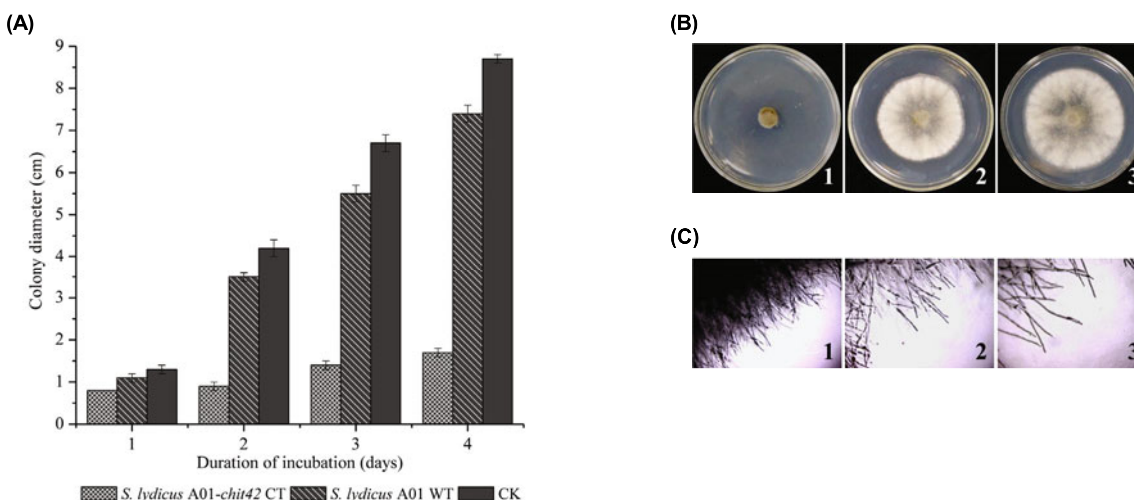

Fig. 8. Control effects of *S. lydicus* A01-*chit42* CT and WT on mycelial growth of *B. cinerea*. (A) Statistic analysis of the colony diameters of *B. cinerea* incubated on PDA plates with fermentation supernatants of *S. lydicus* A01-*chit42* CT, WT, and CK, respectively. (B) Photos showing the colony diameters of *B. cinerea* after a 3 d incubation on PDA plates with fermentation supernatants of *S. lydicus* A01-*chit42* CT, WT, and CK. 1, *S. lydicus* A01-*chit42* CT; 2, WT and 3, CK. (C) Micrographs showing the mycelial morphology of *B. cinerea* on PDA plates with fermentation supernatants of *S. lydicus* A01-*chit42* CT, WT, and CK. Lanes: 1, *S. lydicus* A01-*chit42* CT; 2, WT and 3, CK.

compared with the WT, *S. lydicus* A01-*chit42* CT produced much more chitinase, which could degrade chitin (the solo carbon source) into glucose, further supporting its faster cell growth and denser mycelial development along with higher natamycin production.

The improvements in chitinase activity and natamycin production indicated that the control effect of *S. lydicus* A01-*chit42* CT on *B. cinerea* could be substantially improved by simultaneous chitinase decomposition of the fungal cell wall and natamycin impacts of the growth of fungal cell membranes. Results from the antagonistic tests showed that *S. lydicus* A01-*chit42* CT seriously inhibited *B. cinerea* in terms of spore germination and mycelial growth. In addition, it obviously changed the mycelial morphology of *B. cinerea*. The control effect of *S. lydicus* A01-*chit42* CT on *B. cinerea* was substantially strengthened as compared with the WT (Figs. 7 and 8). During infection, the *B. cinerea* spore first forms a germ tube, adherent cell and invasive nail, which penetrate into leaves and/or fruits, and then continues to infect plants with mycelial elongation. Therefore, field application of the fermentation supernatant of *S. lydicus* A01-*chit42* CT may prevent *B. cinerea* infection by inhibiting spore germination and/or preventing mycelial elongation, further barring the infection.

Further to the resistance to *B. cinerea*, *S. lydicus* A01-*chit42* CT has an advantage regarding chitin powder utilization. During the fermentation process, *S. lydicus* A01-*chit42* CT directly used chitin powder as the sole carbon resource, whereas most other biocontrol agent strains, such as *Streptomyces* sp. M-20 and *Trichoderma harzianum* SQR-T37, may need extra nutrients (at least colloidal chitin) for mycelial growth and chitinase production (Kim *et al*., 2003; Huang *et al*., 2011). Therefore, *S. lydicus* A01-*chit42* CT is potentially useful for degradation of industrial wastes such as shells from crab and shrimp, thus yielding greater economic benefits than most other biocontrol agent strains.

In this study, we selected a simple broth for chitinase expression that contained chitin powder as the sole carbon source and ammonium nitrate as sole nitrogen source. Although *S. lydicus* A01-*chit42* CT produced more chitinase and natamycin than *S. lydicus* A01 WT under laboratory conditions (Figs. 5 and 6), the current experimental production scale is unlikely to meet agricultural requirements. To enhance chitinase and natamycin production and to meet the agricultural and industrial requirements, modification of the recipe of the fermentation broth by supplementing with a certain amount of nutrients, such as yeast extract and peptone, may be considered (Nawani *et al*., 2005). We could also optimize the initial pH value of the broth, the incubation temperature and the rotation speed during the fermentation process (Nampoothiri *et al*., 2004). In addition, the advantages of transformants may further be developed by co-transfer of multiple chitinase genes, even chitinase and other extra-cellular enzymes such as protease and glucanase, to *S. lydicus* A01 (Steyaert, 2004).

Conclusions

In this study, the chitinase-encoding *chit42* gene was highly expressed in *S. lydicus* A01, enhancing the associated natamycin production. Compared with the WT, *S. lydicus* A01-*chit42* CT showed a stronger control effect on *B. cinerea*, primarily due to simultaneous inhibition to spore germination and mycelial growth. To the best of our knowledge, this study represents the first research to transform a *chit42* gene from *T. harzianum* P1 into *S. lydicus* A01 for improving its control effect on *B. cinerea*. Our work demonstrates the potential of producing high-quality biocontrol agents by transforming exogenous resistance genes between organisms with complementary anti-fungal functions.

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