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The Endochitinase of *Clonostachys Rosea* Enhances the Biocontrol Efficiency of *Bacillus Amyloliquefaciens* by Increasing Its Activities of Defense Enzymes

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Abstract

Objectives

To investigate whether the ech42 gene in Clonostachys rosea can improve the biocontrol efficacy of Bacillus amyloliquefaciens and its molecular mechanism.

Results

Compared to the wild type, the B. amyloliquefaciens transformed with the ech42 gene exhibited a higher chitinase activity. The B. amyloliquefaciens-ech42 also showed a significantly higher biocontrol efficiency against B. cinerea when tomato plants were pre-treated with amyloliquefaciens-ech42. No significant difference of control efficiency was observed between the wild type and amyloliquefaciens-ech42 when tomato plants were first infected by B. cinerea. In addition, the activity of the defense-related enzyme polyphenol oxidase, but not superoxide dismutase was significantly higher in amyloliquefaciens-ech42 than in the wild type.

Conclusions

The ech42 enhances the Bacillus amyloliquefaciens biocontrol efficiency by increasing the capacity of protection/prevention to plants, rather treating/killing the pathogens.

Keywords: *Bacillus amyloliquefaciens*, Chitinase, Defense enzymes; Biological control

Introduction

Biological control (biocontrol) of phytopathogens provides an attractive alternative means of management of plant diseases. It is highly efficient and causes no harm to the environment or to

human health. The fungus *Clonostachys rosea* has been tested successfully as a biocontrol species against many plant pathogenic fungi and has shown great potential in controlling plant diseases and promoting crop growth (Keinath et al. 1991; Keyser et al. 2016; Linthorst et al. 1990; Orakc et al. 2010; Schöneberg et al. 2015).*C. rosea* inhibits the growth of pathogenic fungi mainly by producing extracellular lytic enzymes, especially chitinase, which can degrade chitin, the main component of fungal cell wall (Chang 2003; Manuel and Robert 1994; Barrett 2002)

The antifungal effect of chitinase was first reported by Horikoshi and Iida, who found that *Bacillus circulans* exerts lytic activity to *Aspergillus oryzae* and addition of chitinase increases the lytic activity (Horikoshi and Iida 1959). Over-expression of the chitinase gene (chit36 or chit42 of *Trichoderma harzianum* and cht42 of *T. virens*) enhanced the inhibition to *Botrytis cinerea* (Limón et al. 2004; Viterbo et al. 2001; Baek et al. 1999; Wu et al. 2013).

Another well-studied biocontrol agent is *Bacillus amyloliquefaciens*, which inhibits plant pathogens by producing low molecular weight antibiotics and other active substances such as antibacterial polypeptides. *B. amyloliquefaciens* can also promote plant growth as root bacteria (Chen et al.2007). Isolation of chitinases from *B. amyloliquefaciens* has also been reported(Wang et al.2002; Siti et al. 2002), and these chitinases displayed antifungal activities.

In this study, in an effort to further enhance the antifungal activity of *B. amyloliquefaciens*, the endochitinase gene of *C. rosea* (encoded by ech42), was transformed into *B. amyloliquefaciens*. The control effects in vitro against *B. cinerea* were investigated and our results provided evidences of substantially improved inhibitory effects on *B. cinerea*.

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Materials and methods

1. Strains and culture conditions

Bacterial and fungal strains used in this study were listed in Table 1. *B. amyloliquefaciens* and *C. rosea* were cultured in Luria broth (LB) medium (1% tryptone, 1% NaCl and 0.5% yeast extract) at 37°C. Fungi were grown on potato dextrose agar (PDA) plate (20% potatoes (sliced washed unpeeled), 2% dextrose and 2% agar power).

2. Construction of ech42 plasmid

C. rosea was cultured in PDA plate. RNA was extracted and reverse transcribed into cDNA, which was used as the DNA template. The primers for *C. rosea* ech42 gene were designed based on the sequence from the GenBank: DQ523687. The ech42 gene was amplified by polymerase chain reaction (PCR). The PCR mixture includes: 1 μl of forward primer (10 μM), 1 μl of reverse primer, 5 μL of 10×EasyTaq Buffer (TransGen Biotech), 0.5 μL of EasyTaq DNA Polymerase (TransGen Biotech), 4 μL of 2.5 mM dNTPs, 1 μL of cDNA template and 37.5 μL of distilled water. The PCR conditions were consisted of initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products corresponding to the expected molecular size were extracted from the gels and purified using a StarPrep Gel Extraction Kit (Kangrun Biotech), according to the manufacturer' instruction. Both the PCR products and the expression vector pHT43 (MoBiTec GmbH, Germany) were digested with restriction enzymes *Xba* I and *Sma* I (ThermoFisher Scientific, Waltham, Massachusetts) then linked with T4 DNA ligase (ThermoFisher) at 22°C overnight. Consequently, the recombinant plasmid pHT43-ech42

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was constructed transformed into *E. coli* DH5α for gene amplification. The plasmid DNA was isolated and verified by DNA sequencing (Huada Shenzhen, China).

3. Transformation of ech42 gene to *B. amyloliquefaciens*

B. amyloliquefaciens was cultured at 37°C in growth medium (1% peptone, 0.5% yeast extract, 1% NaCl and 9.1 % sorbitol) to a final OD of 0.85. The cell culture was cooled on ice for 10 min, then carefully harvested by centrifugation at 5,000 g and 4℃ for 5 min. The cells were washed four times with ice cold electroporation medium (9% sorbitol, 9.25% mannitol and 10% glycerin), and suspended in the same solution. For transformation, the electro-competent cells were mixed with column-purified recombinant plasmid DNA and loaded into prechilled 1 mm gap electroporation cuvettes. After incubation for 2 min, the cell-DNA mixtures was shocked by 12.5 kv/cm, 15 kv/cm, 17.5 kv/cm, 20 kv/cm or 21 kv/cm voltage, respectively using a GenePulser electroporator (Bio-Rad GenePulser Xcell) with the resistance set at 200 Ω, resulting in a time constant of 4.5-5.0 ms. After that, cells were immediately diluted into 1 ml recovery medium (1% peptone, 0.5% yeast extract, 1% NaCl, 9% sorbitol and 7% mannitol) and incubated at 37℃ for 3 h to allow expression of the antibiotic resistant gene, and aliquots of the dilutions were then spread onto LB agar plates supplemented with 5 μg/ml chloramphenicol.

4. Detection of the plasmids stability and the growth curves of *B. amyloliquefaciens* and the recombinant strain.

The wild type *B. amyloliquefaciens* and the recombinant strain (*B. amyloliquefaciens*-ech42) were inoculated in LB liquid medium with or without 5 μg/ml chloramphenicol. The culture was continued for 30 consecutive generations. The stability of the plasmid was tested once every 10

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generations. After cultured 10, 20 and 30 generations in the antibiotic-free and antibiotic-containing LB liquid medium, the cultures were diluted and plated on LB solid medium without antibiotics. 100 plaques were picked after the colonies were grown and transferred to the LB solid medium containing antibiotics. The number of colonies were counted and the colony-forming unit (CFU) was compared to indicate the stability of the plasmid. The growth rates of the two strains were tested by growing them in 100 ml LB liquid medium at 37℃ on a shaker (180 rpm). The optical density (OD) was measured every 2 h for 32 h using an RS232 PRINT spectrophotometer (Nanjing Kaidi High-Speed Analytical company; Nanjing, China) at a wavelength of 630 nm.

5. SDS-PAGE assay

The molecular weight of the ech42 protein was detected by SDS-PAGE. The wild type *B. amyloliquefaciens* and the recombinant strain *B. amyloliquefaciens*-ech42 were incubated in LB liquid medium at 37°C with 1 mM of IPTG for 8 h. 0.4 mg/ml lysozyme (Coolaber, Beijing) was added for lysis of cells. Samples were boiled for 10 min with 2.5% (W/V) SDS. Proteins were separated by electrophoresis, gels were stained with Coomassie blue. Protein molecular weight marker (14,400) was used as a standard to calculate the molecular mass.

6. Determination of chitinase activity

Chitinase activity was determined using the 3,5-Dinitrosalicylic acid (DNS) assay. The quantity of reducing sugar was calculated based on comparison with a standard curve generated from known concentrations of N-acetylglucosamine (0-1 mg/ml). 1 mM of IPTG was added to the culture

medium of *B. amyloliquefaciens*-ech42 and incubated on a rotary shaker (180 r/min) at 37°C for 2 h, 4 h, 8 h, 12 h, and 24 h, respectively. Cells were centrifuged at 8,000 g, 25°C for 1 min and 500 µl of supernatant was added to 1.0 ml of 1% colloida chitin (Solarbio, Beijing). The mixture was incubated at 37℃ for 1 h, and then terminated by the addition of 1.5 ml DNS (Coolaber, Beijing). The reaction was boiled in a water bath for 10 min, then cooled to 25℃. Next, the sample was centrifuged at 8,000 g for 5min and the volume was adjusted to 10 ml with distilled water. The absorbance was measured using a RS232 PRINT spectrophotometer (Nanjing Kaidi High-Speed Analytical company; Nanjing, China) at 540 nm. The experiment was performed in triplicate.

7. Tomato plant growth experiment

To test whether the *B. amyloliquefaciens*-ech42 strain has an enhanced effect against plant pathogens, *B. cinerea*, which causes gray mold disease, was used in a greenhouse setting with tomato plants. The homozygous tomato variety 08016 (provided by the Tomato Research Institute of Northeast Agricultural University in China) were grown in a greenhouse at 25℃/22℃ (day/night) with 16 h light and 8 h dark cycles for 12 weeks. The *B. cinerea* strain was grown at 25℃ on PDA plate. The spore suspensions of the B. cinerea obtained from surface of the 7-day old cultures and suspended in 5 mL of sterile distilled water containing 6.7 mM KH2PO4, 0.1 M glucose, 0.1% Tween, pH 5, and then filtered through four layers of sterile cheesecloth to remove any adhering mycelia. Conidial suspensions were diluted to a concentration of 1 X 10^7 spores mL^{-1} with sterile water as described above. Two experiments, prevention and disease treatment, were conducted in this study: In the prevention experiment, the tomato plants were first treated with the wild type *B. amyloliquefaciens* or the *B. amyloliquefaciens*-ech42 by spraying with a

water bottle. The plants were then treated with *B. cinerea* strain after waiting for 24 h. For treatment experiment, 8 weeks of tomato seedlings were first treated with *B. cinerea* for 24 h. The seedlings were then sprayed with the wild type or the recombinant *B. amyloliquefaciens*. The control seedlings were sprayed with sterile water. 31 fruits were tested for each of the three groups (wild type, recombinant and control). Disease severity was recorded after 7 days of treatment using a 9-grade scoring system based on the area of lesions on tomato leaves as follows: 0, no symptoms; 1, <6%; 3, 6–10%; 5, 11–25%; 7, 26–50%; 9, >50%. The disease index was calculated using the following formula:

Disease index = [Σ(number of infected leaves × disease grade)/(total number of leaves × the highest disease grade)] × 100, and the control efficiency of each treatment was evaluated. Four replicates were performed for each sample.

8. Defense-related enzyme (PPO and SOD) activity assays

Polyphenol oxidase (PPO) activity was measured according to Lee and Park [13, 14], with minor modification.

To assay PPO activity, 1 g of fresh leaves were pulverized in 10 mL of buffer containing 0.24 g polyvinyl polypyrrolidone (PVPP) and 0.1 M sodium phosphate (pH 7.0) in an ice bath. A total of 6 groups of leaves were collected, including leaves treated with the wild type and recombinant bacteria and control (treated with water) from both prevention and treatment experiments. The pulverized leaves were centrifuged at 10,000 g for 10 min at 4℃, and the supernatant was used for enzyme activity assays. A 20 μL aliquot of the supernatant was mixed with 1 mL of a solution containing 0. 4 ml solution A (7.10 g Na_2HPO_4, 5.25g Citric acid, 200 ml distilled water) and 0.1

ml solution B (2.76 g Catechol，50 ml distilled water) and incubated for 10 min at 30℃. PPO activity was measured using a spectrophotometer at 420 nm. One unit of PPO activity is defined as an increase in absorbance of 0.01.

To assay the superoxide dismutase (SOD) activity, 0.5 g of fresh leaves (same as above experiments) were placed in a pre-cooled mortar, and 1 ml of phosphate buffer was gradually added to the grinding process. Additional buffer was added to make the total volume to 5 ml. The grinded leaves were centrifuged at 10,000 g for 10 min at 4℃. A 0.05 mL aliquot of the supernatant was mixed with 3.25 ml of the reaction solution （13 mM Met, 75 μM NBT, 10 μM EDTA-Na^2 and 0.2 μM Riboflavin). Phosphate buffer was used as control. SOD activity was measured using a spectrophotometer at 560 nm. One unit of PPO activity is defined as an increase in absorbance of 0.01.

9. Statistical analysis

Fisher's LSD test was used to compare the significance. A p value < 0.05 was considered as statistically significant.

Results

1. Construction of pHT43-ech42

Digestion of the plasmid pHT43-ech42 by Xbal I and Sma I showed that the pHT43 vector and the ech42 gene were about 7,000 bp and 1,300 bp respectively, which matched the expected sizes. The results of single and double digestion showed that the recombination vectors of pTH43-ech42 was

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constructed successfully (Fig. 1).

2. Transformation of ech42 gene to *B. amyloliquefaciens*

For electroporation transformation, different voltages were used to test the transformation efficiency. The highest number of transformants was obtained at 15kv/cm and no transformants were obtained at 20 kv / cm, 21 kv/cm, probably due to the increased of cell death.

3. Plasmid stability in recombinant *B. amyloliquefaciens* and the growth curves

Plasmid stability test showed that that the transformation of ech42 was stable even after 30 generations of culture. The number of CFU showed no difference when plated on LB medium without chloramphenicol and a medium with chloramphenicol (Fig. 2). The growth rate of recombinant strain (*B. amyloliquefaciens*-ech42) was slower than the wild type (*B. amyloliquefaciens*). The difference of growth rates gradually increased after 14 h culture and reached the maximum after 24 h (Fig. 3).

4. Detection of recombinant ech42 protein by SDS-PAGE

SDS-PAGE showed a 42 kDa protein band in *B. amyloliquefaciens*-ech42 in the presence of IPTG. However, no similar band was observed in *B. amyloliquefaciens*-ech42 in the absence of IPTG or in *B. amyloliquefaciens* induced with IPTG (Fig. 4). The 42 kDa band is similar to the molecular mass of ech42, indicating that the ech42 was successfully expressed in the *B. amyloliquefaciens*-ech42 strain.

5. Chitinase activity of *B. amyloliquefaciens* and *B. amyloliquefaciens*-ech42

Chitinase activity assay showed that the *B. amyloliquefaciens-ech42* displayed a significantly higher chitinase activity than the wild type strain. The activity of *B. amyloliquefaciens*-ech42 was highest at the 8 h incubation (0.156 ± 0.012 U/ml), which was 1.54 times higher than that of the wild type strain (0.101 ± 0.014 U/ml) (Fig. 5). The activity of the wild type was highest at 12 h (0.133 ± 0.007 U/ml), 1.13 lower than the activity of *B. amyloliquefaciens-ech42* (0.15 ± 0.003 U/ml) at the same time point.

6. Control efficiency of *B. amyloliquefaciens*-ech42 against *B. cinerea* in greenhouse

The control efficiency of *B. amyloliquefaciens*-ech42 against *B. cinerea* was further tested using tomato plants in a greenhouse setting. In the prevention experiment (Figs. 6a, 6b), the *B. amyloliquefaciens*-ech42 exhibited a significant higher control efficiency, compared to the wild type *B. amyloliquefaciens* at both day 15 and day 20 ($p < 0.05$). However, in the treatment experiment, no significant differences in control efficiency were observed between the wild type and *B. amyloliquefaciens*-ech42 (Figs. 6c, 6d) ($p > 0.05$). These results suggest that the increased chitinase activity in *B. amyloliquefaciens* works more efficient in plant pathogen prevention than pathogen treatment.

7. Changes in defense-related enzyme activity

During the prevention and treatment experiments, fresh leaves were collected from all treatment to test the enzyme activities of both PPO and SOD. In both the prevention and treatment experiments,

the activities of PPO with *B. amyloliquefaciens-ech42* were significantly higher than that of *B. amyloliquefaciens* or the control day 3-5 for prevention and day 7 for treatment) (Figs. 7a, 7c). The SOD activities of both the wild type and *B. amyloliquefaciens-ech42* significantly higher than that of the control, but no significant differences were observed between the wild type and *B. amyloliquefaciens*-ech42 (Figs. 7b, 7d).

Discussion

Due to the negative impacts of chemical fungicides to the environment, and induction of pathogen resistance, biocontrol is becoming more and more attractive for plant disease management. One of the challenges for biocontrol is to screen or identify more efficient biocontrol agents. Several studies have attempted to improve the antifungal activity against pathogens through improvements in the antibiotic production via ultraviolet mutation, chemical mutagenesis, and/or intraspecific protoplast fusion. Others have shown that chitinase could enhance the inhibition of many plant pathogens (Limón et al. 2004; Viterbo et al. 2001; Baek et al. 1999; Wu et al. 2013). In this study, we transformed the endochitinase gene ech42 isolated from *C. rosea* into the biocontrol fungus *B. amyloliquefaciens*. We then compared the biocontrol efficiency between the wild type and the *B. amyloliquefaciens* overexpressing the chitinase. Consistent with previous studies, we showed that the increased chitinase in *B. amyloliquefaciens*-ech42 is effective to inhibit the growth of several plant pathogens. We also showed that *B. amyloliquefaciens*-ech42 is more efficient in plant pathogen prevention than pathogen treatment.

The results showed that the transformation efficiency of pHT43-ech42 depends on the applied

voltages. The highest number of transformants was obtained with 15kv/cm and no transformants at 20 kv/cm or 21 kv/cm. This is consistent with a previous study, which showed the transformation frequency was at 12 kV/cm, and subsequently, transformation frequency was reduced with increased voltage, probably due to increase of cell death (Shen et al.2013).

In this study, we found that the growth rate of *B. amyloliquefaciens*-ech42 was lower than that of wild type. In contrast, a previous study showed that the growth rate of a recombinant strain was higher than that of wild type of *Streptomyces sp*. They speculated that overexpression of chitinase could degrade chitin (the solo carbon source) into glucose, further supporting the cell growth (Wu et al. 2013).

To further understand the molecular mechanism of the ech42 enhanced biocontrol effect, we investigated the activities of two defense-related enzymes, PPO and SOD. PPO is an oxidase and catalyzes the oxidation of monophenols and o-diphenols to o-diquinones, which can ultimately produce brown or red pigments that cause fruit browning. PPO also plays important roles in disease resistance. It has been shown that overexpression of PPO increased resistance to the bacterial pathogen, *Pseudomonas syringae* (Li and Steffens 2002). SOD acts as an antioxidant that catalyzes the dismutation of reactive oxygen species, which cause oxidative stress and damages to cells (Hayyan et al. 2016). In this study, we found that the PPO and SOD activities of *B. amyloliquefaciens*-ech42 were significantly higher than that of the wild type, when tomato plants were treated first with *B. amyloliquefaciens*-ech42 (prevention experiment). However, when the plants were treated first with the plant pathogen *C. rosea*, the PPO and SOD activities were not

significantly higher. Our results strongly suggest that ech42 enhances the biocontrol efficiency by increasing the capacity of protection/prevention to plants, rather treating/killing the pathogens. As a result, a good strategy for effective biocontrol is to apply the biocontrol agents as early as possible to boost the "immune system" of plants.

One limitation of our study is that our results are not confirmative whether the enhanced biocontrol efficiency of the recombinant *B. amyloliquefaciens* (*B. amyloliquefaciens*-ech42) is caused primarily by the increased level of chitnase. It is possible that the insertion of ech42 may cause other changes to *B. amyloliquefaciens*. Studies using purified chitinase is desirable to further understand the biocontrol mechanism of *B. amyloliquefaciens.*

In conclusion, our study demonstrated that insertion of the *C. rosea* endochitinase gene ech42 into *B. amyloliquefaciens* significantly enhanced the chitinase activity and its biocontrol efficiency. Our results suggest that the increased biocontrol efficiency may be caused by increased capacity of protection/prevention to plants, rather treating/killing the pathogens.

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