Strategies for enhancing the control effect of chitinolytic microorganisms against plant diseases

Seur Kee Park

Department of Agricultural Biology, Sunchon National University

Chitin is an important structural component of fungal cell walls, insect cuticles and crustaceans shells. Therefore, fungi, insects and crustaceans have been shown to produce chitinases for growth of their self, while chitinases from viruses, bacteria, plants and humans contribute to inhibition of fungal pathogens or insects. Thus, some bicontrol agents have been reported from chitinolytic microorganisms. We have conducted studies to increase the specific chitinase activity and the chitinase-productive ability using genetic engineering techniques, and also improve the control effects of chitinolytic microorganisms against several plant diseases. Their strategies and results are introduced in this presentation.

I. Strategies for enhancement of specific chitinase activity and chitinase-productive ability

We found that biocontrol activity of a chitinolytic bacterium, *Chromobacterium* sp. strain C-61, against soilborne plant pathogens is depend on its chitinase-productive ability, indicating that higher chitinase activity or chitinase-productive ability may increase the biocontrol activity. Thus, genes with the enhanced chitinase activity were obtained by modification of a chitinase gene that isolated from *Chromobacterium* sp. strain C-61. A strain that can produce more chitinases was obtained by introduction of the chitinase gene into other chitinolytic strain.

1. Characteristics of a chitinase gene from *Chromobacterium* sp. strain C-61.

A chitinase gene (*chi54*) from strain C-61 was composed of 1,611 nucleotides, which encoded a signal sequence of N-terminal 26 amino acids and a mature protein of 510 amino acids. The protein encoded was calculated to have a molecular mass of 55,102 Da and a pI of 8.67. The pI value was highest among the bacterial chitinases known so far. The *chi54* encoded a protein that included a chitin-binding domain (ChtBD) and a catalytic domain (CatD). The *E. coli* harboring *chi54* secreted more chitinases than wild type strain. The recombinant Chi54 could be purified by a single step using Rotofor cell (Bio-Rad). The optimum pH for activity of purified Chi54 was

pH 7.0 on 4-MU-$(GlcNAc)_2$ and colloidal chitin but pH 10.0 on crystal chitin. The stability was highest at pH 10.0. The temperature optimum of the Chi54 was 50°C and it was stable up to 40°C.

2. Development of genes with enhanced chitinase activity and pathogen-inhibitory ability.

Enhancement of chitinase activity by attachment of cellulose-binding domain:

The chitin-binding domain (ChtBD) in the 3'-prime region of the *chi54* was classified as Group A(ChtBD3a) of the Type 3 (ChBD3). The ChtBD3a of Chi54 was bound to both chitin and cellulose. A family II cellulose-binding domain (CBDIIa) of endoglucanase A from *Cellulomonas fimi* was also shown to bind to both cellulose and chitin. To compare roles or effects of ChtBD3a and CBDIIa on binding affinities and chitinase activity, the ChtBD3a of Chi54 was deleted or replaced by a CBDIIa, The ChtBDs has been generally shown to bind to crystal chitin, and then to increase activity toward the chitin. Thus, the CBDIIa was constructed upstream of ChtBD3a in order to enhance the chitinase activity. These results showed that (1) removal of ChtBD3a remarkedly decrease the chitinase activity, (2) ChtBD3a and CBDIIa have similar functions in the binding affinities and the chitinase activity, and (3) the chitinase activity and binding affinity are enhanced by double domain of ChtBD3a and CBDIIa.

Enhancement of chitinase activity by site-directed mutagenesis:

The catalytic domain (CaTD) in the 5'-prime region of the *chi54* was classified as subfamily A of family 18 chitinases. The members of subfamily A contained chitinases from viruses, bacteria, insects, crustaceans and plants. Their CaTDs consisted of many nonconserved amino residues and some conserved amino residues. Mutation of the conserved residues lost almost chitinase activity, but mutation of the nonconserved residues showed various chitinase activity. Interestingly, mutation of Thr218 to Ser (T218S) increased the chitinolytic ability. Thus, the Thr218 was replaced by the amino residues present in other chitinases; Ala(T218A), Asp(T218D) and Glu(T218E). The chitinase activity increased about 1.5 fold in T218S, but decreased with levels of 1/2 in T218A, 1/5 in T218D and 1/3 in T218E relative to wild type strain. The enhanced activity of the T218S was higher in K_{cat} value. Enhancement of this specific activity increased the inhibitory effect against *Botrytis cinerea*.

3. Development of a transgenic microorganism with enhanced chitinase-productive ability and biocontrol activity

The filamentous fungus *Trichoderma atroviridae* P1 is a potent mycoparasite, which

has been applied as a biocontrol agent against several plant pathogenic fungi. This strain was shown to control plant diseases by combination of antibiotics and chitinolytic enzymes. We hypothesized that more chitinases in the *T. atroviridae* P1 may be produced by expression of the *chi54,* and thus its biocontrol activity may increase. Thus, the *chi54* was constructed downstream signal sequence and promoter of *N*-acetyl-β-D-glucosaminidase gene *nag1,* and then transformed in *T. atroviridae* P1. The transgenic *T. atroviridae* P1 secreted chitinases of itself and *Chromobacterium* sp (Chi54). Its chitinase activity was highest in 5 days cultivation and total chitinase activity increased about 1.5-fold. The control effect of the strain against wilt disease of hot-pepper also increased as comparison with that of wild type.

II. Strategies for practical use of chitinolytic microorganisms in biocontrol of plant diseases.

More important for practical use of biocontrol agents may be to improve their control effect. In our lab, improvement of the control effect has been attempted by combination of chitinolytic microorganisms and chitin, because the chitin is their main nutrient, and also its derivatives inhibit plant pathogens and induce resistance of plant.

1. Biocontrol effect of the nursery soil containing chitinolytic microorganisms and chitin.

We hypothesized as follows; (1) chitinolytic microorganisms, *Trichoderma atroviridae* P1 and *Chromobacterium* sp. may retain good density in the nursery soil containing chitin, because they use chitin as a main nutrient. (2) hot-pepper seedling cultivated in this soil may show high control effect against soilborne pathogens in the field, because they can protect at least main roots. We had seen that plants can live, if main roots are health. However, the density of two strains in the nursery soils+chitin decreased largely when the strains cultivated in PDA or NB broth were directly inoculated. Thus, the strains cultivated in the oatmeal+soil were mixed with the nursery soils+chitin. This method retained better density than direct inoculation. Their densities were also higher in soils containing more amounts of chitin, but level of 1/10 in rate of chitin/soil (w/w) inhibited growth of hot-pepper. Thus, seeds of hot-pepper were planted and cultivated in the nursery soil that contains each strain and chitin of 1/100, and then were transplanted in field. This method increased the growth and inhibited the wilt disease of hot-pepper. Their control effects were better in soil containing *Trichoderma* than *Chromobacterium* sp.

2. Improvement of biocontrol effect by culture solution of chitinolytic microorganisms cultivated in chitin-supplemented medium

We found that chitinase and chito-oligosaccharides, especially pentamer and hexamer, inhibit fungal plant pathogens. Some fungal pathogens were also inhibited by chitin-oligosaccharides. The chitinase and chitin-oligomers can be present in the culture solution of chitinolytic microorganisms when they are cultivated in the chitin-supplemented medium. The culture solutions can also contain unknown antifungal substances. It is suggested that the culture solution may have higher control effect than the chitinolytic bacteria alone. Application of the culture solutions can also offer good conditions to plant pathogens. Thus, we first chose a medium which the antagonists grow well but the pathogens can not grow. In that medium, density of chitinolytic microorganisms and chitinases activity were highest in 7～10-day-culture at 28°C. These culture solutions showed higher control effects against powdery mildew of cucumber than did bacterial cells alone. Thus, mass culture solution was obtained from a large incubator with capacity of 500L and applied in several plants. Their control effects are as follows.

Powdery mildew of Cucumber:

The control effect was higher in culture solution of *Lysobacter enzymogenensis* than in that of *Chromobacterium* sp. strain C-61. The combined culture solution of two strains showed higher control effect than single solution of each strain. The 1/10 dilution of combined culture solution also showed high control value of 93.7%.

Anthracnose and Alternaria blight of Ginseng:

These diseases were to a certain extent controlled by eradication of dead plants and interception of rain. Under these conditions, the combined culture solution of *L. enzymogenensis* and *Chromobacterium* sp. was sprayed on ginseng leaves four times on Jun 3, June 12, July 4, and July 25. Its control effect was very superior, with control values of 81.3% for anthracnose and 84.7% for alternaria blight. These control values were similar with those of fungicidal treatment.

Damping-off and Grey mold of Ginseng:

On PDA plates, the growth of *R. solani* was largely inhibited by *Streptomyces* sp. *Chromobacterium* sp. and *L. enzymogenensis,* and inhibition of *Pythium* sp. was best in *Serratia plymuthica*. The growth of *Botrytis cinerea* was inhibited by all of these strains. However, *S. plymuthica* was interfere with the emergence of ginseng and formed lesion on the ginseng leaves. Thus, three strains of *Streptomyces* sp. *Chromobacterium*

sp. and *L. enzymogenensis* were cultivated together. The soil treatments of the culture solution were conducted two times on December (transplanting time) and on April for control of damping-off and three times on April 30, May 14 and June 3 for control of gray mold. These treatments showed high control values of about 82.3% for damping-off, which was superior to that of Captan treatment, and the control value of 72.8% for gray mold.

Wilt disease of Hot-pepper:

The wilt disease of hot-pepper is caused by interactions of several soil-borne plant pathogens such as *Phytophthora capsici, Rhizoctonia solani, Fusarium oxysporium* and *Fusarium solani*. On PDA plates, growth of *Ph. capsici* was inhibited largely by *S. plymuthica*. While *Rh. solani* was inhibited by *Chromobacterium* sp. and *L. enzymogenes*, and *F. oxysporium* and *F. solani* was weakly inhibited by only *L. enzymogenes*. Thus, combined culture solution of *S. plymuthica, Chromobacterium* sp. and *L. enzymogenes* were drenched in soil three times on June 12, July 4, and July 25. This treatment showed high control value of 91.8%.