行政院國家科學委員會專題研究計畫成果報告

桿菌屬拮抗細菌之開發與改良

Development and modification of *Bacillus* antagonist

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一 、 中 英 文 摘 要

已知許多細菌可產生幾丁質分解酵素分解真菌細胞壁，抑制真菌的生長，故被應用於植物真菌性病害的防治上。本研究將 *Bacillus circulans chiA* 基因導入拮抗細菌 *Bacillus subtilis* 細胞中，測試其對拮抗菌抑制病原真菌生長的影響。首先將含起動子的 *chiA* 基因次選殖於 shuttle vector pHY300PLK 上，以原生質體轉形法送入 *B. subtilis* 細胞中。可於 *B. subtilis* 的培養上清液中測得幾丁質分解酵素的活性，顯示 ChiA1 蛋白質可分泌於 *B. subtilis* 的細胞外。*B. subtilis* 於 LB 中培養 24 小時後可以測得幾丁質分解酵素的活性；在幾丁質培養基中則需培養較長時間才可測得幾丁質分解酵素的活性。在大腸桿菌細胞中，ChiA1 蛋白質主要分泌於細胞間質中。當培養時間延長時，可於幾丁質培養基上菌落外緣出現透明圈。將 *B. circulans chiA* 基因表現於無抑菌作用的變異株，可以觀察到 ChiA 對 *Botrytis elliptica* 的生長具有抑制效果；但幾丁質分解酵素 ChiA1 並不能穩定地促進 *B. subtilis* 野生菌株的抑菌效果。

Several chitinase - producing bacteria are known to degrade the fungal cell wall and are able to inhibit the growth of fungi. Applications of chitinase-producing bacteria in plant disease control have been reported. In this study, we transferred the *chiA* gene of chitinase-producing bacterium, *Bacillus circulans*, into the antagonist, *Bacillus subtilis* and examined the effect of ChiA1 protein on the antagonistic activity of *B. subtilis*. Firstly, the *chiA* gene was subcloned with its own promoter into the shuttle vector, pHY300PLK, and transferred into the bacterial cells of *Bacillus subtilis* by protoplast transformation. The expression of ChiA1 protein was detected in the supernatant of *B. subtilis* culture, indicating that the ChiA1

protein is secreted out of the *Bacillus* cell. The ChiA1 protein was detected 24 hr after cultivation of *Bacillus* in LB broth and in a later period when incubated in chitin medium. In *Escherichia coli* cells, the ChiA1 protein is translocated into the periplasmic space. The chitinase is released from *E. coli* cells after prolonged incubation. The expression of *chiA* gene in a non-antagonistic mutant of *B. subtilis* could display inhibition effect on the growth of *Botrytis elliptica*. However, when the *chiA* gene was expressed in the antagonistic strain of *B. subtilis*, the enhancing effect of ChiA1 on the antagonistic activity of *B. subtilis* was not readily observed.

二、計畫緣由與目的

The antagonistic activity of biocontrol agent could be due to the antabiosis, competition of nutrition, and parasitism. Some antagonistic bacteria are capable of producing chitinases which are involved in the biocontrol of fungal pathogens (Inbar and Chet, 1991; Kobayashi *et al.*, 1995; Ordentlich *et al.* 1988; Pleban *et al.*, 1997; Sneh, 1981). Chitinases are able to degrade the chitin components in the cell wall of fungi (Horikoshi and Iida, 1959). Chitin, a polymer of *N*-acetyl-D-glucosamin in beta- 1,4 -linkage, is one of the components of cell wall of pathogenic fungi in Ascomycetes, Basidiomycetes, and Deuteromycetes. Antifungal activity of chitinase from chitinolytic bacteria is reported in many cases (Chernin *et al.*, 1995; Mavingui and Heulin, 1994). Chitinolytic bacteria or chitinase has been used with antagonistic organism to enhance its toxicity, such that the ChiA1 protein of *Bacillus circulans* could enhance the toxicity of *Bacillus thuringiensis* subsp. *kurstaki* toward diamondback moth larvaes (Wiwat *et al.*, 1996). Endochitinase ChiAII of *Serratia marcescens* has a synergistic effect on the activity of CryIC endotoxin of *Bacillus thuringiensis* toward *Spodoptera littoralis* larves (Regev *et al.*, 1996).

In the genus *Bacillus*, *B. circulans* (Tanaka and Watanabe, 1995; Watanabe *et al.*, 1990), *B. cereus* (Pleban *et al.*, 1997), and *B. polymyxa* (Mavingui *et al.*, 1994) are well known to produce chitinase. Most of *Bacillus subtilis* are not chitinase-producers, many are able to produce antibiotics and are good biocontrol agent for fungal diseases of plants (Aldrich and Bader, 1970, Asaka and Shoda, 1996; Baker and Stavely, 1985, Berger *et al.*, 1996, Tchen, 1987; Podile *et al.* 1985). In this study, we examined the effect of chitinase on the antagonistic activity of *B. subtilis* with the *chiA* gene of *B. circulans*.

二、　結果與討論

The 2.4-kb *chiA* gene of *B. circulans* was cloned into the vector

pCR2.1 and subcloned into the shuttle vector, pHY300PLK (4.8 kb). The clone was designated pNTU111. Clear zone was observed around the colony of *E. coli* DH5α(pNTU111) on chitin medium after 7-day incubation. *E. coli* DH5α(pHY300PLK) hardly grew on chitin medium. In SDS-PAGE analysis, the activity of chitinase was detected in the periplasmic fraction of *E. coil* cells.

The activity of chitinase was detected in the supernatant of *B. subtilis* 28-6(pNTU111) which incubated in LB, nHA and chitin medium. In LB broth, the chitinase was detected significantly 24 hr after inoculation and the activity still could be detected 48, and 96 hr after inoculation. During these incubation periods, chitinase activity was not detected in the culture of *B. subtilis* 28-6 及 *B. subtilis* 28-6(pHY300PLK). The chitinase activity appeared about one day delayed when *B. subtilis* was cultured in nHA medium and two days delayed in chitin medium. In addition, the chitinase collected from the supernatant of *B. subtilis* 28-6(pNTU111) could significantly release the fluorescence compound, 4-MU, from 4-MU-$(GlcNAc)_3$.

The *chiA* fragment amplified in this study includes its own promoter and a 2,097-bp open reading frame for a protein product, ChiA1 (Watanabe *et al.*, 1990). Although the expression of *chiA* is inducible in *B. circulans* (Watanabe *et al.*, 1990), it is consistently expressed in both *E. coli* and *B. subtilis* when *chiA* was constructed in the shuttle vector, pHY300PLK. The constitutive expression of *chiA* in *B. subtilis* may be explained by the possibilities that a repression control in *B. circulans* does not present in *B. subtilis* and *E. coli* or another upstream promoter on the vector may drive the expression of *chiA* gene.

By using nHA medium for the growth of target fungi and antagonist, *B. subtilis* 28-6 (pNTU111) showed stronger inhibition effect on the growth of *Rhizoctonia solani* AG4 than *B. subtilis* 28-6(pHY300PLK); however, this effect could not reproduced regularly. This inconsistency might be due to the states for the production of antibiotic and chitinase were not well controlled. The instability of pNTU111 in *B. subtilis* 28-6 was observed under a non-selective condition. Thus, a test was performed in the medium containing tetracycline for selection of plasmid. This phenomenon will become a problem in pot assay. An improvement on this point will be considered. Plasmid pHY300PLK have been reported stable in *Bacillus thuringiensis* and still a stable construct with a chitinase gene of *Aeromonas* sp. (Wiwat *et al.*, 1996). Although plasmid pHY300PLK was also stable in *B. subtilis*, the construct was not as stable with the *chiA* gene of *B. circulans* in a non-selective condition.

To clarify the effect of chitinase expressed in *Bacillus subtilis* on the growth of target fungi, the *chiA* gene was introduced into a *B. subtilis* mutant

fenE which could not inhibit the growth of *Rhizoctonia*. By using weak malt extract agar for the growth of target fungi and chitin medium for the growth of antagonist, *B. subtilis* fenE (pNTU111) showed stronger inhibition effect on the growth of *B. elliptica* than *B. subtilis* fenE (pHY300PLK), indicating that chitinase ChiA1 could inhibit the growth of *B. elliptica.* Similar effect was not observed on *B. cinerea*, *Rhizoctonia solani* AG-1, and *Fusarium oxysporum*. The reason is not clear.

四、成果自評

We showed that *chiA* gene of *B. circulans* could inhibit some pathogenic fungi when the gene was introduced into a *B. subtilis* strain. The enhancing effect of ChiA protein on the antagonistic activity of *B. subtilis* had been observed; but not consistently displayed. The interactions of ChiA1 protein of *B. circulans* and antibiotics of *B. subtilis* may exist. The expression of *chiA* gene and antibiotic synthetase genes within same *Bacillus subtilis* cell may be counteracted. A better way to use chitinase gene in plant disease control will be put in deep consideration.

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