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A Efficient Selection of Hybrids Following Intergeneric Transfer of Nuclei from *Trichoderma harzianum* into *Gliocladium virens* Protoplasts

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*Gliocladium virens*와 *Trichoderma harzianum*의 屬間 核 轉移體의 效率的 選拔

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ABSTRACT: To obtain hybrids producing antagonisms and plant growth promoting effects by intergeneric nuclei transfer, the nuclei were isolated from the protoplasts of *Trichoderma harzianum* T95 and treated with colchicine. The nuclei were tranferred into protoplast of multi-auxotrophic *Gliocladium virens* G88 which cannot grow in minimal medium. The nuclei transferred into protoplasts of *G. virens* G88 were selected on the regeneration minimal medium containing chloroneb as a haploid inducer. Low transfer frequency of 0.08% was observed with three chemical treatment, however no segregants were found in the intergeneric nuclei transfer. The various types of hybrids with different morphology were detected when different concentration of chloroneb were treated. These morphologies were classified as parental, recombinant and *petite* type.

KEYWORDS: Intergeneric nuclei transfer, *Trichoderma*, *Gliocladium*, Colchicine, Chloroneb

Introduction

Gliocladium and *Trichoderma* have effectively been used for control of several soilborne plant pathogen by production of extracellular enzymes and secondary metabolites (Papavizas, 1985; Ridout *et al.*, 1986; Robert and Lumsden, 1990). Another interesting aspect of the *Trichoderma* spp. and *Gliocladium* is the plant growth-promoting effect. Especially, growth responses induced by *Trichoderma* spp. appear to be due to both the control of minor pathogen and production of growth-regulating factors (Baker, 1989). Protoplast fusion and the incorporation of prokaryotic DNA into eukaryotes can be a useful genetic manipulation tool for the integrated biocontrol of plant pathogen (Kirimura *et al.*, 1988; Shin and Cho, 1993). Recently, fungal protoplast fusion has been estabilished as a technique for transfer of genetic material, and it provides an effective method for strain improvement (Peberdy and Frenczy, 1989). Intergeneric

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protoplast fusion have been carried out in a few industrial fungi in order to obtain overproducts of secondary metabolites. However, the results were shown to be either very imbalanced heterokaryons or homokaryons that differed markedly from the parental strains (Chang *et al.*,1982). This is primarily because a) lossless of physiological active substances by auxotrophs, and b) hybrids formed diploid due to segregated into both parent by continuous culture. To resolve above problems, transfer of isolated cytoplasmic genetic elements (e.g., nuclei or mitochondria) of wild type into protoplasts may provide a novel means for complementation of genetic information, which may be important for genetic studies on filamentous fungi especially species lacking a sexual stage. Unfortunately, segragation into parent by conventional nuclei transfer would not to be solved. In this communication, we report a efficient technique for genetic transfer in filamentous fungi succeed in solving a segregation of hybrids by treatment of chemical reagents.

Materials and Methods

Fungal strains and media

Multi-auxotrophic mutant *G. virens* G88 cannot grow in minimal medium selected from *G. virens* wild type was used for nuclear transfer as described previously (Shin and Cho, 1993). *Trichoderma harzianum* T95 was kindly provided by Prof. R. Baker from Colorado State University (Ahmad). The fungi were grown and maintained on complete medium (CM) containing Yeast extract 10 g, glucose 30 g, casamino acids 5 g, peptone 4 g, sucrose 20 g, KH_2PO_4 0.46 g, K_2HPO_4 1 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g per liter. Minimal medium (MM) containing glucose 20 g, KH_2PO_4 0.46 g, K_2HPO_4 1 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g per liter. The regeneration complete medium (RCM) and regeneration minimal medium (RMM) were prepared by adding 0.6 M sorbitol to CM and MM.

Isolation of nuclei and nuclei transfer

Isolation of nuclei from protoplasts of *T. harzianum* T95 were carried out as described by Yoo *et al.* (1987) and Sivan *et al.* (1990). The isolated protoplasts were harvested by filtration through sintered glass filter (porosity 1). The protoplasts diluted with 5 m*l* of nuclei isolation medium (NIM) containing 0.3 M sorbitol, 5 mM $MgSO_4 \cdot 7H_2O$, 10 mM $CaCl_2$, 20% glycerol, 7% ficoll 400 and triton X-100 and homogenized for 3 min in Ten Broeck Glaso Tissue Homogenizer (Corning Glaso Works, Corning, N. Y.) to lyse the protoplast. The homogenized suspension was filtered sequently through Millipore (Pore size, 8.0 μm) to remove remaining protoplasts. The filtrate was centrifuged at 1000 g for 15 min to sediment nuclei but not smaller organells. Isolated nuclei were recovered by sucrose gradient. Intergeneric nuclei transfer from *T. harzianum* into *G. virens* G88 was followed by the procedure of Yoo *et al.* (1987). The nuclei suspended in 1 m*l* NIM and 15 μg/m*l* colchicine, were centrifuged at 1000×g and the nuclei (ca. 3×10^{10}) was mixed with 1×10^8 to 3×10^8 protoplasts of *G. virens* G88 suspended in 0.5 m*l* of 0.6 M sorbitol and phosphate buffer (pH 6.5). The mixture was amended with 500 μ*l* of a solution containing 60% (Wt/Vol) PEG (M.W. 3550), 10 mM $CaCl_2$, and 50 mM glycine (pH 8.0).

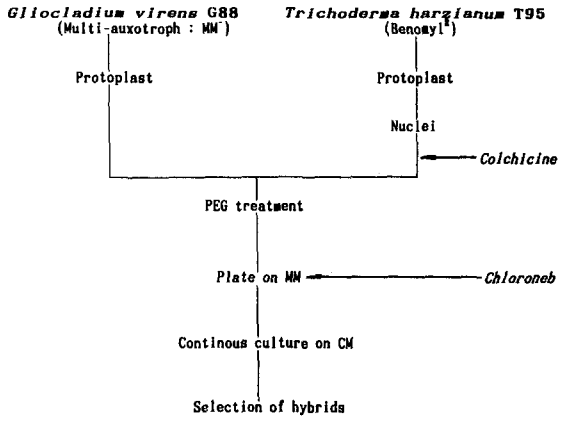

Fig. 1. Strategy for efficient selection of hybrids following intergeneric transfer of nuclei from *Gliocladium virens* and *Trichoderma harzianum.*
MM⁻: *Gliocladium virens* G88 cannot grow in minimal medium, Benomyl^R: benomyl resistant (10 μg/m*l*), Colchicine: 15 μg/m*l*, Chloroneb: 2-20 μg/m*l*.

The PEG mixture was added to the protoplast-nuclei suspension by gentle rolling the tube. The hybrid mixture was incubated at 30℃ for 15 min, and nuclei and protoplasts were forced together by centrifugation for 5 min at 1000×g. This pellet was resuspended in 5 m*l* osmotic stabilizer. Serial dilutions of treated protoplasts were plated onto 0.6 M sorbitol stabilized RMM and RCM supplemented 2-20 μg/m*l* chloroneb.

Selection of intergeneric hybrids

After *T. harzianum* nuclei transfer into *G. virens* G88, colonies growing in RMM were tranferred to MM to confirm their prototrophy. The selected colonies were cultured continously for maintaining genetic stability and analyzed various morphological features.

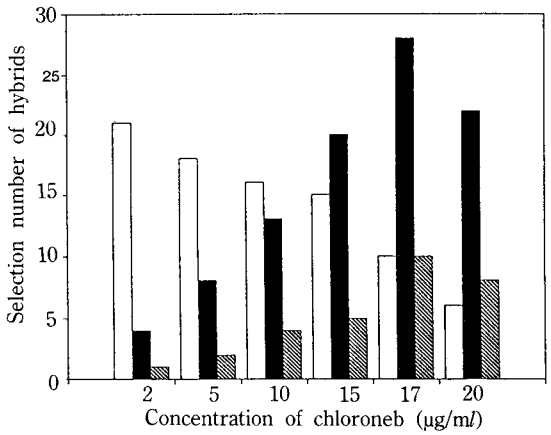

Fig. 2. Effect of concentration of chloroneb in selection of hybrids.
□, *T. harzianum*-like morphologies; ■, *G. virens*-like morphologies; ▨, non-parental or aneuploid formal hybrids.

Results and Discussion

The isolated nuclei from protoplasts of *T. harzianum* T95 were treated with colchicine at concentration of 15 μg/m*l* and transferred into the protoplasts of multi-auxotrophic *G. virens* G88. The hybrids were produced by treating with haploid inducer chloroneb (e.g., $C_8H_8Cl_2O_2$, 1,4-dichloro-2, 5-dimethoxybenzen; chlorophenyl fungicide) on the regeneration minimal medium (Fig. 1). This method was used to obtain hybrids of various morphologies from unusal mendelian model by treatment of mannual chemical reagent. As shown in Fig. 2, the selected hybrids were revealed differently in concentration of chloroneb. Among selected hybids, *T. harzianum*-like morphologies were appeared dominantly with the lower concentration of chloroneb. On the contrary, *G. virens*-like morphologies and recombinants were dominant when high concentration of chloroneb was used. It demonstrated that the concentration of chloroneb was important in selection of various morphological hybrids. The transfer frequency was 8×10^{-3} based on the ratio of the number of colonies formed on RMM and RCM. It revealed that transfer frequency of treatment with colchicine and chloroneb treatment was lower than that untreated on. So far we have obtained 52 hybrids classifible as three type of parent-like morphologies, recombinants, and *petite* (Fig. 3). As to first type, 24 hybrids formed colonies like *G. virens* or *T. harzianum*. They grew more rapidly or slowly than the prototrophic parent. As to second type, 18 hybrids formed colonies showing recombinants on CM. They grew more fastly than *G. virens*. Those hybrids were assumed to be non-parental strains such as heterokaryons or aneuploids. As to third type, one hybrid revealed rare, small, very slow-growing, abnormal colony. The hybrids were judged to be segregants cannot selected. From the above results, the intergeneric transfer frequency was lower with colchicine and chlorneb treatment than untreated one but segregants were not detected. And various morphologies of hybrids were selected with different concentration of chlorneb. Intergeneric transfer technique was found to be a powerful tool in the construction of intergeneric hybrids in imperfect strains of *Gliocladium* and *Trichoderma* or other fungi lacking of sexual stage.

적 요

길항작용 뿐만 아니라 식물생장촉진효과가 있는 속간 핵 전이체를 선발하기 위하여 *T. harzianum*의 원형질체로부터 핵을 분리한 다음 15 μg/m*l*의 콜히친을 처리하였다. 최소배지에서 성장하지 않은 영

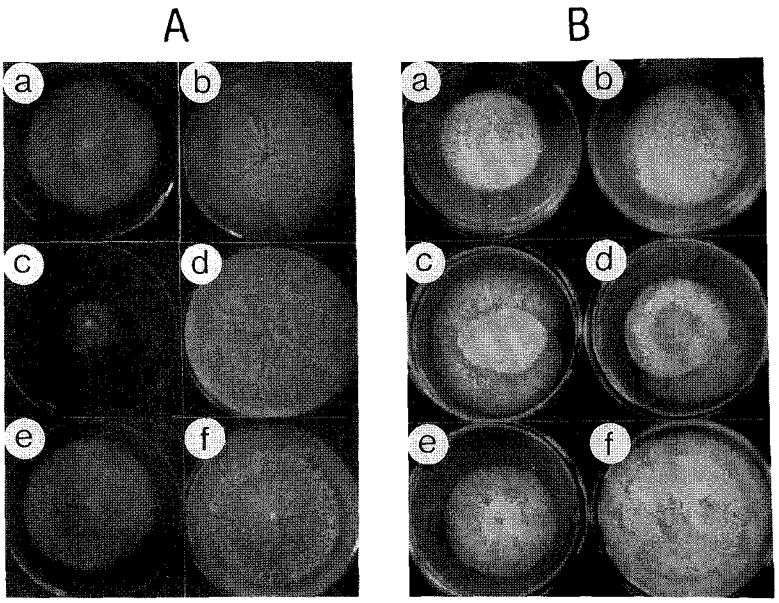

Fig. 3. Comparison of morphology of hybrids from intergeneric transfer by treatment of colchicine and chloroneb (A) and without (B).
a, *T. harzianum* T95; b, *G. virens* G88; c to f, hybrids. Colonies growing in MM supplemented 2-20 μg/m*l* chloroneb were cultured continously on CM.

양요구성 균주인 *G. virens* G88의 원형질체에 콜히친이 처리된 핵을 전이하여 chloroneb이 함유된 재생용 최소배지에서 선발하였다. 전이효율은 0.08%로서 콜히친 및 chloroneb을 처리하지 않은 것보다는 낮지만 segregants는 전혀 나타나지 않았다. 또한 chloroneb 농도에 따라 다양한 형태의 전이체가 선발되었으며 재조합형, 양친형, 그리고 *petite*형 등으로 동정되었다.

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