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Molecular Characterization of Intergeneric Hybrids between *Trichoderma harzianum* and *Gliocladium virens*

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Nuclei were isolated from the protoplasts of *Trichoderma harzianum* T95 and treated with colchicine, a polyploid inducer. The nuclei were transferred into the protoplast of multi-auxotrophic *Gliocladium virens* G88 which cannot grow in minimal medium. The protoplast of *G. virens* G88 carrying the transferred nuclei were regenerated in a regeneration minimal medium containing 17 μg/ml of chloroneb as a haploid inducer. Six intergeneric hybrids between *G. virens* and *T. harzianum* were isolated from the regeneration minimal medium. The hybrids could be classified into three types according to morphology, those with an isozyme pattern, those with an protein band and those with an randomly amplified polymorphic DNA(RAPD) pattern produced by random primers and repetitive sequences. The first group was identified to be a haploid recombinant, the second group a heterokaryon, and the third appeared to be *petite*.

Biological control of soilborne plant pathogens by the application of antagonistic microorganisms has become a major subject in the area of plant pathology. *Gliocladium virens*, one of the most promising fungal biocontrol agents has been used against a wide range of plant pathogens including *Pythium ultimum* (23), *Rhizotonia solani* (9, 14), *Sclerotium rolfsii* (22) and *Gliocladium virens* (10) suppresses the target pathogens by producing antibiotic metabolites such as gliotoxin and gliovirin or by producing hydrolytic enzymes like chitinase or β-1,3-glucanase (10, 15). It has been reported that some of the agents may also produce growth regulating substances which stimulate plant growth.

Trichoderma harzianum, which is closely related to *G. virens*, has also been reported to enhance plant growth by suppressing plant pathogens and/or producing plant growth-promoting substances (3, 8, 16). The plant growth promoting effect of *T. harzianum* is weaker than that of *G. virens*, however, the strain is more rhizosphere competent than *G. virens* (1, 17).

Genetic manipulation of biocontrol agents offers a possible avenue for the improvement of their biocontrol activity against plant pathogens. Recently, protoplast fusion or the transfer of isolated nuclei between intergeneric fungi has been performed to obtain heterokaryons, heterodiploids and recombinants. Intra- and interspecific protoplast fusion in *Trichoderma* sp. and intergeneric protoplast fusion between *Penicillum* and *Cephalosporium*, *Aspergillus* and *Trichoderma*, and *Trichoderma* and *Gliocladium* have been demonstrated successfully (6, 11, 24, 27). However, the fusion resulted in a low complementation frequency and fusion progenies were shown to be either very imbalanced heterokaryons or homokaryons that differed markedly from the parental strains. Transfer of isolated cytoplasmic genetic elements such as nuclei, mitochondria and plasmids into protoplasts may provide a novel means for genetic complementation. Nevertheless there are few reports of nuclei transfer within filamentous fungi including yeast (4, 7), *Trichoderma* (26), and other higher fungi (31, 32).

With that in mind we engineered an intergeneric hybrid between *G. virens* and *T. harzianum* by nuclei transfer with the goal/intention of isolating effective biocontrol agents carrying the most benificial traits from both parents. This report discusses the morphological, biochemical and molecular biological characteristics of the intergeneric hybrids obtained by intergeneric nuclear transfer from *T. harzianum* to *G. virens*.

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MATERALS AND METHODS

Fungal Isolates and Media

Gliocladium virens G872 was isolated from an infected soil at Gyeongsang National University. *Trichoderma harzianum* T95 was kindly provided by Prof. R. Baker of Colorado State Universit (Ahmad). Mutant of *G. virens* G872, G88, which can grow in complete medium (CM) but cannot thrive in minimal medium (MM) was screened by UV-mutagenesis as described by Peberdy and Ferenczy (19). CM contained yeast extract 10 g, glucose 30 g, casamino acids 5 g, peptone 4 g, sucrose 20 g, KH_2PO_4 0.46 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g per liter. MM contained 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 both CM and MM, respectively.

Isolation of Nuclei and Intergeneric Nuclear Transfer

Protoplasts were isolated from *G. virens* mutant G88 and *T. harzianum* T95 according to the methods of Shin and Cho (24) by the incubation of CM-grown mycelium treated with cell wall lytic enzyme containing 0.6 M sorbitol and 10 mM phosphate buffer (pH 6.5) for 3 h at 30°C on CM plate. Protoplasts were purified from the residual mycelial debris by filtration through a sintered glass filter (porosity 1) and washed twice with 0.6 M sorbitol with centrifugation for 10 min at $100 \times g$. The washed protoplasts were resuspended in 0.6 M sorbitol and plated on the RCM or RMM for regeneration. The number of protoplasts was counted under a microscope with illumination using a haemocytometer. Isolation of nuclei from protoplasts and intergeneric nuclei transfer from *T. harzianum* T95 to the protoplast of *G. virens* G88 was carried out by the procedure of Sivan *et al.* (26) and You *et al.* (31, 32) with minor modifications. The nuclei (ca. 3×10^{10}) isolated from protoplasts of *T. harzianum* T95 were treated with 15 μg/ml colchicine, a polyploid inducer, and mixed with $1\sim3 \times 10^8$ protoplasts of *G. virens* G88 (25). The mixture was plated on RCM and RMM containing 17 μg/ml chlorneb, a haploid inducing agent.

Selection of Intergeneric Hybrids

After intergeneric nuclei transfer from *T. harzianum* T 95 to *G. virens* G88, colonies growing in RMM were transferred to MM to confirm their prototrophy. Analysis of spontaneous segregation of progenies on MM and CM was carried out and putative hybrids were selected according to their growth in MM and their morphological features.

Isolation of Fungal Metabolites

The parental and hybrid fungi were grown in potato dextrose broth by saking. The culture suspesions of fungi were filtered using two layers of filter paper. Filtrates were extracted with chloroform and then allowed to completely dry under vaccum. The residues were dissolved in a small volume of methanol and diluted 10 times with distilled water used for bioassays.

Assay of Antifungal Activity

Antifungal activity of the extracted fungal metabolites against hyphal growth was assayed under sterile conditions as described by Howel and Stipanovic (10). Mycelia plugs from the actively growing fungi plates were placed on the center of plates containing PDA medium. After 20~24 h incubation of the petri plates at room temperature to allow spore germination and vegetative growth, sterile paper filter discs were laid on the agar surface and 10 μl of the chloroform extracted from the fungal metabolite was applied to the discs. The plates were then incubated at room temperature for 20 h and growth inhibition of phytopathogenic fungi was observed.

Bioassay of Plant Growth Promoting Substance

The biological effect on plant growth promotion of metabolites produced by the parental and intergeneric hybrids of the fungi was tested using the wheat leaf unrolling index as described by Wada *et al.* (28). The seeds of wheat (*Triticum aesativum* L.cv Keuroo-Mil) were germinated on a wet cotton sheat and grown in darkness at 28°C for 6~7 days. Leaf segments (1.5 cm long) were excised from a region of 1.5 cm below the apex. The segments were incubated in 5 ml of H_2O containing fluorescent bands separated by TLC. Serial dilution of TLC of the one unit of absorption maximum A_{243} was 10^{-7} to 10^{-4}. The width of unrolled leaf segments was measured with calipers.

Detection of Isozymes and Total Soluble Proteins

The isozyme pattern of hybrids was tested by discontinuous polyacrylamide gel electrophoresis with the enzymes esterase, peroxidase, chitinase, and carbonate dehydratase. For isozyme visualization, the gels were tested according to the methods used by Brewer (5) and Ridout *et al.* (21). Total soluble proteins were analyzed by SDS-PAGE and the discontinuous buffer method as described by Laemmli (12).

Fungal DNA Isolation and PCR

Total genomic DNA was isolated from parental or intergeneric hybrids as described by Raeder and Broda (20). DNA samples were then purified through CsCl/ethidium bromide preparative ultracentrifugation at $350{,}000 \times g$ for 4 h. For the RAPD reactions, 15 ng of total DNA in 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.02% gelatin, 200 mM of each dNTP (Phamacia) with 0.2 mM of each primers (GTCGATGTCG, CTGAAGCGGA, ATGTTCCAGG, $(GTG)_5$, $(GT)_8$, and $(CCTA)_5$) and 0.5 units of AmpliTaq (Promega) in a final volume of 50 μl were used. The mixture was covered with 50 μl of mineral oil and the tube was placed in a thermal cycler (Pharmacia) programmed as follows: 30 sec at 94°C and 20°C for 10 min for one cycle; 1 sec at 50°C, 45 sec at

72°C, 5 sec at 94°C and 30 sec at 20°C for 45 cycles; 5 min at 72°C for one cycle.

RESULTS

Hybrid Isolation

The nuclei isolated from protoplasts of *T. harzianum* T95 were treated with the polyploid inducer colchicine at a concentration of 15 μg/ml and then transferred into the protoplasts of multiauxotrophic *G. virens* G88 which cannot grow in MM. The hybrids pretreated with 17 μg/ml of the haploid inducer chloroneb were regenerated on the regeneration minimal medium. Transfer frequency was 0.08%. Transfer frequency was lower when the protoplasts were treated with colchicine and chloroneb than without treatment, but segregants were not observed. Among several hybrids, six hybrids possessing genetic stability were selected for continuous culture.

Analysis of Intergeneric Hybrids

The morphological characteristics of *G. virens* G88, *T. harzianum* T95 and their intergeneric hybrids are three types (Fig. 1). The first type, Fig. 1F, 1G and 1H forms colonies like those *G. virens* G88 on MM and CM. They grew faster than G. virens G88. Those hybrids were assumed to be recombinants. The second type, Fig. 1C and 1D forms colonies like *T. harzianum* T95. They grew as well as the prototrophic parent, *T. harzianum* T95. The hybrids of the second type were judged as heterokaryons like prototrophic *T. harzianum* T95. The third one, Fig. 1E is very slow growing petite type. From the above results, the hybrids selected were of recombinants, heterokaryons and *pertite*.

Isozyme patterns of esterase, peroxidase, chitinase and carbonate dehydratase were compared with those of the parents. Hybrids F2-10 and F2-16 produced a new chinase isozyme with a lower molecular weight in addition to the parent one (Table 1). The hybrids F17-4, F17-5 and F17-11 produced a different size with those of carbonate dehydratase from the parental type. For biocontrol, selected hybrids were tested for antifungal effect against white rot and damping off caused by *R. solani* and *P. ultimum*. The hybrid F2-10 showed the most potent antagonistic effect against plant pathogen, *R. solani*

Table 1. Isozyme patterns of the intergeneric hybrids selected on CM.

Strains	Isozyme phenotype[α]			
	EST	POD	CHI	CDH
Hybrids				
F17-4	G88	G88	G88	β
F17-5	G88	G88	G88	β
F17-11	G88	G88	G88	β
F2-10	T95	T95	β	T95
F2-16	T95	T95	β	T95
F6-1	–	–	–	–

[α]EST, Esterase; POD, Peroxidase; CHI, Chitinase; CDH, Carbonate dehydratase. [β]Different isozyme patterns from that of two parents.

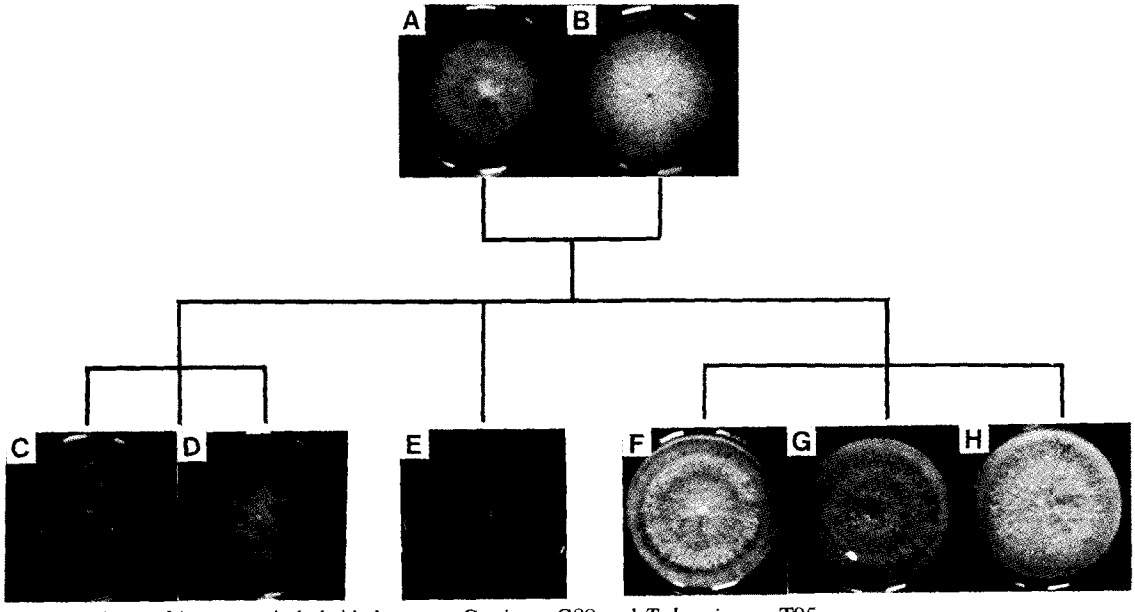

Fig. 1. Morphology of intergeneric hybrids between *G. virens* G88 and *T. harzianum* T95.
Appearance of colonies grown at 27°C for 3 days in MM. A, Parent *T. harzianum* T95 (nuclei donor); B, Parent *G. virens* G88 (nuclei acceptor); C to H, Hybrids (C, F2-10; D, F2-16; E, F6-1; F, F17-5; G, F17-4; H, F17-11).

and *P. ultimum* (Fig. 2). We also examined the plant growth promoting effect of hybrids using the wheat leaf unrolling test. The hybrid F17-5 exhibited a stronger plant growth promoting effect than wild type *G. virens* G88 (Table 2). Electrophoretic profiles of soluble proteins were compared with those of the parents. The soluble protein of the hybrid F2-10 was similar to prototrophic *T. harzianum* T95 but major bands of M.W. 67 and 75 kDa appeared weak (Fig. 3B). The hybrid F17-5 was observed as being different from parent patterns which indicated recombinant type (Fig. 3C).

To confirm the genetic constitution of intergeneric hybrids, these were screened for polymorphic DNA fragments generated by PCR amplification of random DNA sequences using the single 10-mer oligonucleotide primers and simple repetitive sequences as described by Waugh and Powell (29). Polymorphism was detected in the amplification products generated from each parent and their hybrids. RAPD fingerprints of each parent and their hybrids using the primers A, B and C are shown in Fig. 4. All the major fragments amplified by RAPD PCR were reproducible under identical conditions. The primers generated sets of products ranging from 0.5 to 2 kb. The parental and hybrid strains contained differential amplification loci for each primer which were also seen in parental-like or in differential fragment patterns in PCR products. *T. harzianum* T95 and hybrid F2-10 were formed with nearly identical amplification loci for each prim-

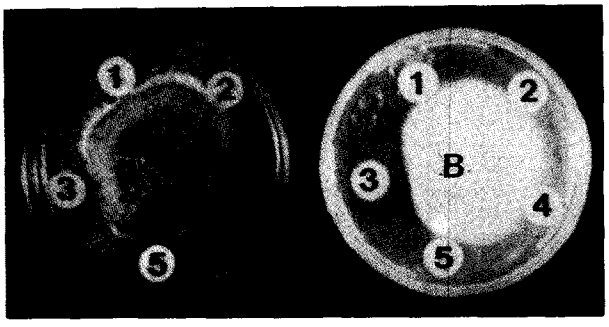

Fig. 2. Antifungal activities of *G. virens* G88, *T. harzianum* T95 and their intergeneric hybrids against *Rhizoctonia solani* (A) and *Phythum ultimum* (B).
The culture extracts of each fungal cell were extracted with chloroform and 10 μl of the extract were spotted on the discs. And then the plates were incubated for 3 days at 27°C. A: 1, negative control (distilled water); 2, F17-5 hybrid; 3, parent *G. virens* G88; 4, F2-10 hybrid; 5, parent *T. harzianum* T95. B: 1, parent *G. virens* G88; 2, negative control (distilled water); 3, F2-10 hybrid; 4, F17-5 hybrid; 5, parent *T. harzianum* T95.

Table 2. Wheat leaf unrolling activity of the culture filtrate[α].

Strains	Leaf segment width (MM) ± Standard error	Unrolling[β]
Control	1.56±0.05	100
G. virens G88	1.78±0.23	161
T. harziamum T95	1.69±0.08	136
Hybrids		
F17-5	2.05±0.25	208
F2-10	1.65±0.11	125

[α]Culture filtrate was extracted with chloroform. The extract was allowed to evaporate and dry and solved in distilled water. [β]Unrolling % was calculated from the initial leaf width (WI), leaf width after treatment (Ws) and width of fully unrolling segment (Wf): (Wf-Wi)/(Ws-Wi)×100.

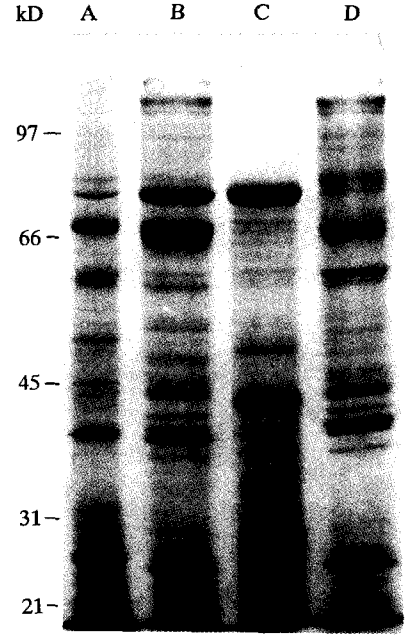

Fig. 3. SDS-polyacrylamide gel electrophoretic pattern of total soluble proteins.
A, Parent *T. harzianum* T95 (nuclei donor); B, F2-10 hybrid; C, F17-5 hybird; D, parent *G. virens* G88 (nuclei acceptor).

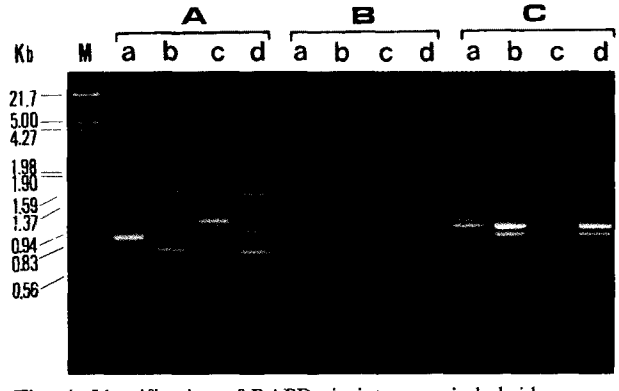

Fig. 4. Identification of RAPDs in intergeneric hybrids.
RAPD generated by oligonucleotide primer GTCGATGTCG (A), CTGAAGCGGA (B) and ATGTTCCAGG (C), analyzed on 2% agarose gel. Lane a, parent *G. virens* G88 (nuclei acceptor); b, Parent *T. harzianum* T95 (nuclei donor); c, F17-5 hybrid; d, F2-10 hybrid.

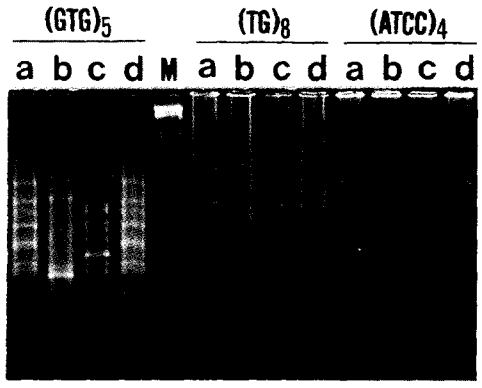

Fig. 5. Screening of intergeneric hybrids by occurance of simple repetitive DNA motifs in genome of fungi.
RAPD generated by simple repetitive DNA sequence primer $(GTG)_5$, $(GT)_8$ and $(CCT)_4$ analyzed on 2% agarose gel. Lane a, parent *G. virens* G88 (nuclei acceptor); b, Parent *T. harzianum* T95 (nuclei donor); c, F17-5 hybrid; d, F2-10 hybrid.

er, but hybid F17-5 was recovered with various fragments such as appearance or loss of RAPD fragments. Using primer A, a band of about 0.9 kb was apparent in a major fragment of *G. virens* G88 which could be differentiated by the presence of a fragment of 1.1 kb in the hybrid F17-5 (Fig. 4A). Fragments of minor bands generated with primer B were present in *G. virens* G88, but were not found in hybrid F17-5. The products amplified from the primer C in F17-5 which were recovered in differential fragments in parental strains. We first attempted to determine whether it was possible to differentiate between parents and their hybrids with a comparative examination of the DNA sequences amplified by PCR with the three different simple repetitive oligonucleotide sequences $(GTG)_5$, $(GT)_8$ and $(ATCC)_4$ according to a modified method of Weising *et al.* (30). Whilst some of the bands were common to all lines, there was similarity to PCR products with 10-mer, not only in the major bands but also in most of the minor fragments. Fig. 5 shows that simple repetitive sequence $(ATCC)_4$ produced a single number of apparently amplified, and lane a and d were appeared to be similar bands. The hybrid F2-10 was revealed that it was originated from *G. virens* G8. But lane c maybe that hybrid F17-5 was formed recombinant. Additionally, the hybrid F6-1 revealed a rare, small, very slow-growing, abnormal colony. This was was not detected in previous hybrid experiments, probably because of its scarcity and the abnormal growth pattern.

DISCUSSION

Protoplast fusion in filamentous fungi has proved to be a highly efficient procedure for obtaining heterokaryons in many species (18). However, it is difficult to select multi-auxotrophic strains, overcome incompatibility, and ensure the stability of secondary metabolites. To overcome this problem, nuclear transfer has been demonstrated in yeast, plants, and filamentous fungi (7, 13, 26, 31). We screened multi-auxotrophic *G. virens* G88 which cannot grow in MM from the *G. virens* wild type lacking a sexual stage to overcome incompatibility, and selected intergeneric hybrids having putative recombinant or heterokaryon properties by subjecting colchicine and chloroneb treatment (25). Novel combinations of nuclear and cytoplasmic genes in partial hybrids result not only in dominance of particular morphological traits of the donor or recipient, but also in the manifestation of traits which are expressed in neither of the parents. The full characterization of hybrids is a prerequisite for an effective exploitation of nuclear transfer in filamentous fungi improvement. The selected hybrids obtained were stronger in terms of antifungal or plant growth promoting activities as compared to the wild type, but did not carry both parental properties. Among several hybrids, F17-5 and F2-10 have been found as effective biocontrol agents as characterized by morphological features, isozyme and protein patterns, RAPD, and simple repetitive sequences. The hybrid F2-10 was also selected as a heterokaryon as follows: i) the morphology of *T. harzianum*, ii) increasing of secondary metabolite (or antifungal activity), iii) protein, RAPD and simple repetitive sequences were similar to *T. harzianum* T95. This indicates that the nuclei of *T. harzianum* T95 was transferred into protoplasts deficient nuclei isolated from *G. virens* G88 mycelia. The hybrid F17-5 was identified as a haploid recombinant with the following characteristics: i) it is a fast-growing macrotransgenomic type, ii) it increase the secondary metabolite a plant growth promoting substance, iii) it has nonparental patterns of soluble protein and amplification fragments as shown by RAPD and simple repetitive sequences. This indicates that a nonparental hybrid may result from karyogamy and genetic recombination, from cytoplasmically inherited characteristics, or from nuclear-cytoplasmic interactions. These results indicate that nuclear techniques are useful for the construction of intergeneric hybrids but that unresolvable problems arose in our experiments. The novel intergeneric hybrids produced should permit the development of a complete set of additional lines such as syncretic hybrids obtain antifungal and pant promoting activities in the future.

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REFERENCES

1. Ahmad, J. S. and R. Baker. 1987. Rhizosphere competence of *Trichoderma harzianum. Phytopathol.* **77**: 182-189.
2. Baird, E., S. Cooper-Bland, R. Waugh, M. DeMaine, and W. Powell. 1992. Molecular characterization of inter- and intra-specific somatic hybrids of potato using randomly amplified polymorphic DNA (RAPD) markers. *Mol. Gen. Genet.* **233**: 469-475.
3. Baker, R. 1989. Improved *Trichoderma* spp. for promoting crop productivity. *TIBTECH* **7**: 34-38.
4. Becher, D., B. Conrad, and F. Bottcher. 1982. Genetic transfer mediated by isolated nuclei in *Sacharomyces cerevisiae. Curr. Genet.* **6**: 163-165.
5. Brewer, G. J. *An introduction to isozyme techniques.* Academic Press, New York.
6. Chang, L. T., D. T. Terasaka, and R. P. Elander. 1982. Protoplast fusion in industrial fungi. *Dev. Ind. Microbiol.* **23**: 21-29.
7. Ferenczy, L. and M. Pesti. 1982. Transfer of isolated nuclei into protoplasts of *Sacharomyces cerevisiae. Curr. Microbiol.* **7**: 157-160.
8. Hadar, Y., I. Chet, and Y. Henis. 1979. Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum. Phytopathol.* **69**: 64-68.
9. Howell, C. R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum, Rhizoctonia solani*, and damping-off of cotton seedlings. *Phytopathol.* **72**: 496-498.
10. Howell, C. R. and R. D. Stipanovic. 1983. Gliovirin, a new antibiotic from *Gloicladium virens*, and its role in the biological control of *Pythium ultimum. Can. J. Microbiol.* **29**: 321-324.
11. Kirimura, K., M. Imura, Y. Kato, S. P. Lee, and S. Uasi. 1988. Intergeneric protoplast fusion between Aspergillus niger and *Trichoderma viride. Agri. Biol. Chem.* **52**: 1327-1329.
12. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head bacteriophage T4. *Nature* **227**: 680-685.
13. Lorz, H. and I. Potrykus. 1978. Investigations on the transfer of isolated nuclei into plant protoplasts. *Theor. Appl. Genet.* **53**: 251-256.
14. Lumseden, R. D. and J. C. Locke. 1989. Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. *Phytopathol.* **79**: 361-366.
15. Lumseden, R. D., J. C. Locke, S. T. Adkins, J. F. Walter, and C. J. Ridout. 1992. Isolation and localization of the antibiotic gliotoxin produced by *Gliocladium virens* from alginate prill in soil and soilless media. *Phytopathol.* **82**: 230-235.
16. Nelson, E. B., G. E. Harman, and G. T. Nash. 1988. Enhencement of *Trichoderma*-induced biological control of *Pythium* seed rot and pre-emergence damping-off of pea. *Soil. Biol. Biochem.* **20**: 145-150.
17. Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. *Ann. Rev. Phytopathol.* **23**: 23-54.
18. Peberdy, J. F. 1989. Presidential address: Fungal without coats-protoplasts as tolls for mycological research. *Mycol. Res.* **93**: 1-20.
19. Peberdy, J. F. and L. Ferenczy. 1985. *Fungal protoplasts: Application in biochemistry and genetics.* Marcel Dekker Inc. New York, Basel.
20. Raeder, U. and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1**: 17-20.
21. Ridout, C. J., J. R. Coley-Smith, and J. M. Lynch. 1986. Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizotocnia solani. J. Gen. Microbiol.* **132**: 2345-2352.
22. Ristaino, J. B., K. B. Perry, and R. D. Lumsden. 1991. Effect of solanization and *Gliocladium virens* on *Sclerotium rolfsii*, soil microbiota, and the incidence of Southern blight of tomato. *Phytopathol.* **81**: 1117-1124.
23. Roberts, D. P. and R. D. Lumsden. 1990. Effect of extracellular from *Gliocladium virens* on germination of sporangia and mycelia growth of *Pythium ultimum. Phytopathol.* **80**: 461-465.
24. Shin, P. G. and M. J. Cho. 1993. Intergeneric protoplast fusion between *Gliocldium virens* and *Trichoderma harzianum. Korean J. Mycol.* **21**: 322-331.
25. Shin, P. G., Y. B. Yoo, J. C. Ryu, Y. H. Park, and M. J. Cho. 1994. A efficient selection of hybrids following intergeneric transfer of nuclei from *Trichoderma harziaum* into *Glicladium virens* protoplasts. *Korean J. Mycol.* **22**: 276-280.
26. Sivan, A., G. E. Harman, and T. E. Stasz. 1990. Transfer of isolated nuclei into protoplast of *Trichoderma harzianum. Appl. Environ. Microbiol.* **56**: 2404-2409.
27. Stasz, T. E., G. E. Harman, and N. F. Weeden. 1988. Protoplast preparation and fusion in two biocontrols of *Trichoderma harzianum. Mycologia* **80**: 141-150.
28. Wada, K., H. Kondo, and S. Marumo. 1985. A simple bioassay for brassinosteroids: a wheat leaf-unrolling test. *Agri. Biol. Chem.* **49**: 2249-2251.
29. Waugh, R. and W. Powell. 1992. Using RAPD markers for crop improvement. *TIBTECH* **10**: 186-191.
30. Weising, K., B. Beyermann, J. Ramser, and G. Kahl. 1991. DNA fingerprinting of *Ascochyta rabiei* with synthetic oligodeoxynucleotides. *Electrophoresis* **12**: 159-169.
31. Yoo, Y. B., P. G. Shin, H. K. Kim, M. O. Byun, C. H. You, D. Y. Cha, and K. Y. Chang. 1989. Transfer of isolated nuclei from *Agrocybe aegerita* mycelia into *Pleurotus florida* protoplasts. *Korean J. Mycol.* **17**: 114-118.
32. Yoo, Y. B., C. H. You, P. G. Shin, Y. H. Park, K. Y. Chang. 1987. Transfer of isolated nuclei from *Pleurotus florida* into protoplasts of *Pleurotus ostretus. Korean J. Mycol* **15**: 250-253.

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