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Interspecific Protoplast Fusion of *Trichoderma koningii* and *Trichoderma reesei*

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*Trichoderma koningii*와 *Trichoderma reesei* 원형질체 융합

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Abstract: Intra and interspecific fusants were produced by the protoplast fusion of auxotrophic mutants from *Trichoderma koningii* ATCC 26113 and *Trichoderma reesei* QM 9414. It was found that 0.6M $MgSO_4$ and 0.6M NH_4Cl was the best osmotic stabilizer for the preparation of protoplasts from the mycelium of *T. koningii and T. reesei* respectively. However, $MgSO_4$ was the most suitable one for the regeneration of the protoplasts from both species. The intraspecific protoplast fusion frequencies between the auxotrophic mutants from *T. reesei* were 1.8×10^{-2} to 5.1×10^{-1}. Interspecific protoplast fusion frequencies between the auxotrophic mutants from *T. koningii* and *T. reesei* were 3.6×10^{-3} to 8.4×10^{-2}. Interspecific complementing fusants, however, were not always produced. Fusants obtained from interspecific potoplast fusion were spontaneously segregated into various strains including parental types, non-parental auxotrophic hybrids, and prototrophic hybrids on complete plate. Interspecific hybrids revealed to have partially enhanced celluloytic activities.

Key Words: protoplast fusion, *Trichoderma koningii*, *Trichoderma reesei*, interspecific hybrid

The genetic study of genus *Trichoderma* has not been done because of its absence of available sexuality. Results of intra and interspecific protoplast fusion obtained in the past ten years clearly indicate the possibility and importance of protoplast fusion as the new method of genetic transfer.

Interspecific complementing hybrids in *Penicillium* (Anné *et al.*, 1976), *Aspergillus* (Kevei and Peberdy, 1977, 1984), and *Verticillium* (Typas, 1983) were successfully isolated. Consequently, this simple and effective method for the high frequency transfer of genetic materials has led to great advances in the genetic research of microorganisms, especially, in which the technique to exchange genetic materials has not been well developed.

We have already reported on the conditions for the preparation and fusion of protoplasts from mycelium and conidiospore (Cho *et al.*, 1981a, b; Park *et al.*, 1983; Hong *et al.*, 1984a, b; Hong and Park, 1985), and on the electronmicroscopic observation of protoplasting and regeneration of protoplasts from mycelium (Lim *et al.*, 1983) and conidiospore (Park *et al.*, 1984) in *Trichoderma koningii*. There are only a few reports, however,

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on the protoplast fusion of industrially important fungus, genus *Trichoderma* (Hong *et al.*, 1984 a, b; Toyama *et al.* 1984 a,b; Hong and Park, 1985).

To elucidate the possibility of strain improvement and transfer of genetic materials through protoplast fusion in genus *Trichoderma*, intraspecific protoplast fusion in *T. reesei*, and interspecific protoplast fusion between *T. koningii* and *T. reesei* were performed. And some properties of complementing intra and interspecific fusants were described.

MATERIALS AND METHODS

Organisms and media

Trichoderma koningii ATCC 26113, *Trichoderma reesei* QM9414 and their auxotrophic mutants obtained by the methods described below were used in these experiments. Complete medium (CM) contained 2% malt extract (Difco), 0.1% Bactopeptone (Difco), and 1% dextrose. Minimal medium (MM) contained 1% dextrose and the mineral components of Medium C (Mandels *et al.*, 1962). Complete regeneration medium (CRM) was the CM supplemented with 0.6M $MgSO_4$. Minimal regeneration medium (MRM) was the MM supplemented with yeast nitrogen base w/o amino acid (0.65%, Difco) and 0.6M $MgSO_4$. For solidifying regeneration medium, 2% agar was autoclaved separately to avoid hydrosis, and Triton X-100 0.05%, v/v) was added to the CM and MM plates for restriction of colony size.

Mutagenesis

Ultraviolet irradiation: Conidiospore suspensions of parental strains (1.0×10^7m*l*) in 0.01M phosphate buffer (pH5.8) were exposed to UV light (8-10 erg/cm_2/sec) with stirring for 25min to kill over 99% of the population.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and 4-nitroquinoline-1-oxide (4-NQO) treatment: Conidiospore suspensions were treated with NTG (0.2mg/m*l*, Sigma) for 50min and treated with 4-NQO (2μg/m*l*, Sigma) for 15min at 30°C to kill over 99% of the population. Survivors were subsequently enriched for auxotrophs by the standard filtration method (Freis 1947). The lineage of the auxotrophs used in these experiments is listed in Table 1.

Preparation and fusion of protoplasts

Preparation and fusion of protoplasts were performed by the previous methods (Hong *et al.*, 1984 a, b).

Segregation of fusants

Colonies of complementing fusants developed on the MRM plates were transferred to CM plates and allowed to segregate spontaneously. After conidiation, conidiospores from the fusants were plated on CM plates to determine auxotrophic requirements.

Induction of cellulase

Conidiospores were inoculated into 20m*l* of medium consisted of 0.6% glycerol, 0.03% yeast extract (Difco), 0.1% peptone (Difco) and the mineral components of the Medium C. The cultures were incubated at 28°C on a rotatory shaker for 48h. The mycelia were harvested, washed twice with the solution of Medium C and used as an inoculum into 100m*l* conical flask containing 20m*l* of induction medium consisted of car-

Table 1. *Lineage of mutants used in these experiments.*

Strain	Growth factor/ Charateristics	Origin	Mutagen
T. koningii			
AF-1	ade	ATCC 26113	UV
A-7	arg	〃	〃
CFT-1	yel, arg	CF-1	NTG
AT-7	arg, leu	A-7	〃
AFT-1	ade, lys	AF-1	〃
CUT 121	whi, lys, met	C-2	〃
T. reesei			
UV-1	lys	QM9414	UV
UV-2	met	〃	〃
UV-3	pro	〃	〃
UQ-21	lys, met	UV-2	4-NQO
UQ-31	lys, pro	UV-3	〃

Abbreviations used are as follows; ade: adenine, arg: arginine, leu: leucine, lys: lysine, met: methionine, pro: proline, whi: white, yel: yellow.

boxymethyl cellulose (CMC; 0.5%, Sigma), Avicel (0.5%, Sigma) and the mineral components of the Medium C. The final mycelial dry weight in induction medium was 150mg. Cultures were incubated at 28°C on a rotatory shaker for 5-6 days. After cultivation, the culture filtrate was used as a crude cellulase source.

Enzyme assay

Carboxymethyl cellulase (CMCase) and Avicelase activity: Each reaction mixture was composed of 0.8m*l* of 0.5% CMC (or Avicel) in 0.05M sodium acetate buffer (pH 5.0) and 0.2m*l* of crude enzyme solution. After incubation at 50°C for 30min (for Avicel for 1h), reducing sugar concentration was determined according to the method of Somogyi (1952). One unit of enzyme activity was expressed as 1μg of reducing sugar liberated per m*l* of reaction mixture under the assay condition.

β-glucosidase activity: β-glucosidase activity was assayed by measuring amounts of p-nitrophenol (PNP, Sigma) liberated from p-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma). Reaction mixture was composed of 0.8ml of 1mM PNPG in 0.05M sodium acetate buffer (pH 5.0) and 0.2m*l* of enzyme solution. After incubation at 40°C for 30min, 2m*l* of 1M sodium carbonate solution was added to the mixture. The mixture was diluted with 10m*l* of distilled water and absorbance at 420nm was measured. One unit of enzyme activity was expressed as 1μmole of PNP liberated per m*l* of reaction mixture under assay condition.

Determination of protein concentration: Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

RESULTS

Isolation and regeneration of protoplasts

Conditions for isolation and regeneration of protoplasts from mycelium of *T. reesei* QM 9414 were investigated. In case of *T. reesei* (results not shown), optimum culture age, concentration of lytic enzyme system, and reaction conditions for

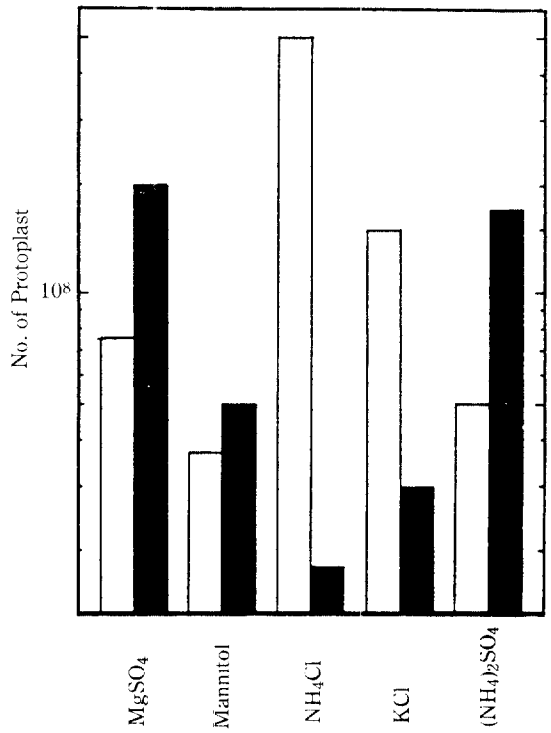

Fig. 1. *Effect of various osmotic stabilizers on the formation of protoplast. T. koningii* ATCC 26113 (■) and *T. reesei* (□) were cultivated for 18h in CM. After cultivation, mycelia were harvested and treated with 1% Driselase in various osmotic stabilizers for 3h at 28°C; The concentration of the salts was 0.6M in 0.01M phosphate buffer, pH 5.5.

the isolation of protoplasts were almost the same as those of *T. koningii* (Cho *et al.*, 1981a,b). However, effective stabilizer system for the isolation of protlasts from the mycelium of *T. reesei* was different from that for *T. koningii*. As shown in Figure 1, 0.6M NH_4Cl was the most effective

Table 2. *Effect of osmotic stabilizers on the regeneration of protoplasts of T. reesei*

Stabilizer[a]	Regeneration frequency
$MgSO_4$	2.7×10^{-2}
NH_4Cl	5.4×10^{-4}
Mannitol	4.1×10^{-5}
$(NH_4)_2SO_4$	7.0×10^{-5}
KCl	1.7×10^{-4}
Sorbitol	below 10^{-7}

[a] each stabilizer was added into complete agar medium to give a final concentration of 0.6M.

Table 3. *Intraspecific fusion frequency crossed between protoplasts from the mycelium of various auxotrophic mutants of T. reesei QM 9414*

Cross (genotype)	No. of colonies on MRM ($\times 10^2$)	No. of colonies on CRM ($\times 10^3$)	Fusion frequency
single × single			
UV-1 (lys) × UV-2 (met)	10	56	1.8×10^{-2}
UV-1 (lys) × UV-3 (pro)	25	69	3.6×10^{-2}
double × double			
UQ-21 (lys, met) × UQ-31 (pro, lys)	102	20	5.1×10^{-1}*

*MRM was supplemented with common growth factor lysine.

one for *T. reesei* in comparison with 0.6M $MgSO_4$ for *T. koningii*. For the regeneration of protoplasts from *T. reesei* on CRM plate supplemented with 0.6M NH_4Cl was lower than that with 0.6M $MgSO_4$ (Table 2).

Intra and interspecific protoplast fusion

Intraspecific protoplast fusion in *T. Reesei*, and interspecific protoplast fusion between *T. Roningii* and *T. Reesei* were performed by PEG-$CaCl_2$ induced fusion (Hong *et al.*, 1984b). Intraspecific complementing fusants were successfully produced through fusion of protoplasts from *T. reesei* QM 9414. The intraspecific fusion frequencies were 1.8×10^{-2} to 5.1×10^{-1} (Table 3). In cross between UQ-21 and UQ-31, complementing fusants were not developed on MRM but on MRM plate supplemented with common growth factor, lysine.

Interspecific complementing fusants were produced through fusion of protoplasts from *T. koningii* and *T. reesei* (Table 4). However, interspecific complementing fusants were not produced in all of the crosses investigated. It was noteworthy that, in cross between AFT-1 and UQ-31 that require common growth factor lysine, complementing prototrophic were produced. The interspecific fusion frequencies were 3.6×10^{-3} to 8.3×10^{-2} in crosses that produced fusants.

Segregation of interspecific complementing fusants

Table 4. *Interspecific fusion frequency crossed between protoplats from the mycelium of various auxotrophic mutants of T. koningii ATCC26113 and T. reesei QM 9414*

Cross (genotype)	No. of colonies on MRM ($\times 10^2$)	No. of colonies on CRM ($\times 10^3$)	Fusion frequency
single × double			
A-7 (arg) × UQ-31 (pro, lys)	37	66	5.6×10^{-2}
AF-1 (ade) × UQ-21 (lys, met)	24	29	8.3×10^{-2}
AF-1 (ade) × UQ-31 (pro, lys)	9	51	1.8×10^{-2}
CFT-1 (arg) × UQ-21 (lys, met)	–	42	–
CFT-1 (arg) × UQ-31 (pro, lys)	–	54	–
double × double			
AT-7 (arg, leu) × UQ-21 (lys, met)	3	83	3.6×10^{-3}
AT-7 (arg, leu) × UQ-31 (pro, lys)	–	32	–
AFT-1 (ade, lys) × UQ-21 (lys, met)	–	20	–
AFT-1 (ade, lys) × UQ-31 (pro, lys)	43	53	8.0×10^{-2}
CUT 121 (lys, met) × UQ-21 (lys, met)	–	33	–
CUT 121 (lys, met) × UQ-31 (pro, lys)	–	16	–

Hyphal tissue from the margine of actively growing colonies of interspecific complementing fusants on the MRM plates were maintained on MM plates for stock culture. We assumed that the complementing fusants were in heterokaryotic state. Therefore, hyphal tissues from the stock cultures and/or from the fusants on MRM plate were transferred onto CM plates and allowed to segregate spontaneously. After conidiation, conidiospores from the fusants were plated on CM plates, and growth requirements of the conidiospores were investigated.

As shown in Table 5, all of the interspecific fusants were segregated into parental, prototrophic, and non-parental auxotrophic types.

Table 5. *Spontaneous segregation of interspecific fusants obtained from protoplast fusion between various auxotrophic mutants of* ***T. koningii*** *ATCC 26113 and T. reesei QM 9414*

Cross (genotype)	Heterokaryons were grown on	No. of colonies tested	Genotype of conidia from heterokaryon				
			pro, lys	ade	arg	ade, lys	proto. rec
UQ-31 (pro, lys) × AF-1 (ade)	MRM	100	3	87		10	–
	MM	132	7	122		3	–
UQ-31 (pro, lys) × A-7 (arg)	MM	176	27		22	17	110
UQ-31 (pro, lys) × AFT-1 (ade, lys)	MRM	125	63			62	

Table 6. *Extracellular cellulase activities of various parental strains of* ***T. koningii*** *ATCC26113 and T. reesei QM 9414 and their hybrids obtained from interspecific protoplast fusion*

Strain (genotype)	Avicelase (units)	CMCase (units)	β-glucodase (units)	Protein (μg/ml)
ATCC 26113 (prototype)	40.47	202.97	690	102.42
QM 9414 (prototype)	73.59	191.72	1950	432.73
AF-1 (ade)	24.84	38.72	650	97.88
UQ-31 (pro, lys)	67.34	226.72	590	311.52
AF-1×UQ-31				
FAQM 133	17.97	180.47	780	119.39
FAQM 241	27.97	262.34	780	117.58
FAQM 108	30.16	232.03	670	88.79

The fusant obtained from the cross between UQ-31 and AFT-1 completely segregated into parental types, thus non-parental auxotrophic and prototrophic segregants were not detected. On the other hand, the complementing fusants from the crosses between UQ-31 and AF-1 were segregated into parental and non-parental prototrophic types, but non-parental auxotrophic types were not detected. The complementing fusant obtained from the cross between UQ-31 and A-7, however, was segregated into parental, non-parental auxotrophic and prototrophic types. Among the interspecific prototrophic hybrids derived from the spontaneous segregation of fusants from the cross between UQ-31 and AF-1, three kinds of hybrids were randomly selected and designated FAQM 108, FAQM 133, and FAQM 241. These hybrids were used for further experiments.

Extracellular cellulase activities of the interspecific hybrids

To elucidate the possibility of strain improvement in the imperfecti fungus, genus *Trichoderma* via protoplast fusion, extracellular cellulase activities of the hybrids were measured. As shown in Table 6, the extracellular cellulase activities were partially enhanced. Extracellular CMCase and β-glucosidase activites of the hybrids, as a whole, were higher than those of parents. Increase in Avicelase activities of hybrids, however, were not detected.

DISCUSSION

Benitez *et al.* (1975) have reported that $MgSO_4$ was the best stabilizer for *Micromonospora chalcea* lytic system, and $(NH_4)_2SO_4$ was efficient with both *M. chalcea* and *Streptomyces venezuela* RA lytic system for isolation of protoplasts from mycelium of *T. viride*. Picataggio *et al.* (1983) have reported that $MgSO_4$ and $(NH_4)_2SO_4$ were effective with Driselase in preparing protoplasts from mycelium of *T. reesei*, however, protoplasts prepared with $MgSO_4$ as osmotic support were difficult to sediment. On the other hand, Toyama *et al.* (1983, 1984a, b) have used sucrose as an osmotic stabilizer with *T. viride* lytic system for isolation of protoplasts from immature conidiospores from *T. reesei*. Recently, Jacobsen *et al.* (1985) have reported that $MgSO_4$ was the best osmotic stabilizer for preparation of protoplasts from mycelium and arthroconidia of *Geotrichum candidum*, whereas, regeneration was faster with

mannitol than with $MgSO_4$. It has also been shown that 0.6M $MgSO_4$ was likewise effective for the regeneration of protoplasts from *T. koningii* (Cho *et al.* 1981a). Therefore, it is suggested that kind of osmotic stabilizer for the preparation of protoplasts is related to kinds of cell wall lytic systems and traget microorganisms used. From these results, it seems that osmotic stabilizer may not only support the osmotic potential of osmotic sensitive protoplasts but also affect the regeneration of protolpasts.

In the intraspecific protoplast fusion between UQ-21 and UQ-31, colonies of the complementing fusants were dectected not on MRM but MRM supplemented with lysine. This result suggests that the mutaion site of both stains, UQ-21 and UQ-31, on the gene concerned with lysine biosynthesis might be same, even though the parental strains of both auxotrophic mutants are not the same one.

Interspecific complementing fusants were not always produced in all of the crosses investigated (Table 4). It suggests that the existenec of certain factor(s) (or gene) concerned with the formation of interspecific fusant. However, it can not the be ruled out that *T. koningii* and *T. reesie* might not be distantly related species in taxonomical relationships. Several authors (Anné, 1982; Kevei and Peberdy, 1977; Sipiczki *et al.* 1982) have reported that heterokaryons were easily produced by interspecific protoplast fusion of closely related species, however, interspecific heterokaryons of distantly related species were scarcely produced.

The results shown in Table 5 indicate that the complementing prototrophic fusants developed on MRM by PEG-induced interpecific protoplast fusion are in heterokaryotic state, and that karyogamy of two parental nuclei occur at certain area of heterokaryotic mycelium. Therefore, during the cultivation of the complementing prototrophic fusants on CM plates, segregation of fused nuclei into parental types, nonparental prototrophic hybrids, and non-parental auxotrophic recombinants could occur. From the above results, it is consequent that exchange of genetic materials in the imperfecti fungus, genus *Trichoderma* could be accomplished via protoplast fusion.

The increase in extracellular cellulase activities of prototrophic hybrids derived from segregation of interspecific complementing fusants proved the possibility of strain improvement of the cellulolytic fungus, genus *Trichoderma*. Recently, intraspecific hybrids of *T. koningii* which showed increased extracellular cellulase activities were also obtained from protoplast fusion in this laboratory (in preparation).

The results reported here indicate that the protoplast fusion technique is a good one for the construction of interspecific hybrids in genus *Trichoderma*, and that the exchange of genetic materials and taxonomical studies of imperfecti fungi can be done with the proptoplast fusion.

적 요

*Trichoderma koningii*와 *Trichoderma reesei*의 영양요구성 돌연변이주들로부터 얻은 원형질체를 융합시킨 결과, 상보적인 융합체가 형성되었다. 원형질체생성과 환원에 미치는 삼투안정제의 효능을 조사한 결과, *T. koningii*의 경우에는 0.6M $MgSO_4$가 *T. reesei*의 경우에는 0.6M NH_4Cl이 원형질체 생성에 효과적인 삼투안정제였다. 원형질체의 환원에는 *T. reesei*의 경우, *T. koningii*의 경우와 마찬가지로 0.6M $MgSO_4$가 가장 우수한 것으로 나타났다. *T. reesei*의 종내 원형질체융합율은 $1.8\times10^{-2}-5.1\times10^{-1}$수준이었으며, 종간 원형질체융합율은 $3.6\times10^{-3}-8.3\times10^{-2}$수준이었다. 원형질체융합 결과 얻어진 종간융합체는 완전평판배지상에서 자연 분리되어, 융합모균과 동일한 영양요구형, 융합모균과는 다른 영양요구형 및 독립영양형의 잡종이 생성되었다. 분리결과 얻어진 독립영양형 종간잡종의 섬유소분해효소능을 조사한 결과, 섬유소분해효소능이 증진된 것으로 나타났다.

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