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Construction of Interspecific Hybrids between *Aspergillus* spp. by Nuclear Transfer†

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수종의 *Aspergillus* 속 균 사이의 핵전이에 의한 종간잡종 형성

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ABSTRACT: Interspecific hybrids between the *Aspergillus* spp., *A. awamori*, *A. usamii* and *A. oryzae*, were obtained by nuclear transfer technique. Nuclei isolated from an auxotrophic mutant strain were transferred into the protoplasts of a recipient strain of different species. The frequency of interspecific hybrid formation by nuclear transfer was $2\times10^{-5}-7\times10^{-4}$. In contrast, no interspecific hybrid was isolated by protoplast fusion. Among the hybrids tested, 10 strains showed increased activity of some or all components of cellulases, xylanases and amylase up to more than two times. Isozyme patterns of the hybrids were analyzed by polyacrylamide gel electrophoresis and isoelectric focusing followed by activity staining, which showed that some of the hybrids have isozyme patterns unidentical to either of the two parents. By measuring the DNA contents and the sizes of the conidia, the karyotypes of the hybrids were estimated to be aneuploid near to haploid, diploid or triploid. It was concluded that the nuclear transfer technique is much more efficient in the formation of interspecific hybrids than protoplast fusion, and is very useful for the improvement of *Aspergillus* strains.

KEY WORDS□ Interspecific hybrid, Nuclear transfer, *Aspergillus*, Cellulase, Xylanase

The fungal strains of genus *Aspergillus* have very effective enzyme systems for the hydrolysis of cellulosic materials. They especially show much higher β-glucosidase, β-xylosidase and xylanase activity than *Trichoderma* spp. that are known to be the most potent cellulolytic organisms (Bisaria and Ghose, 1981; Woodward and Wiseman, 1982). Therefore, it is of economic importance to construct new strains of *Aspergillus* containing increased degrading capability of cellulosic resources composed mainly of cellulose, hemicellulose and lignin.

Since Ferenczy *et al.*, (1975a) reported that the fusion of fungal protoplasts was effectively induced by the presence of polyethylene glycol (PEG), protoplast fusion has been frequently applied for the improvement of fungal strains useful in biotechnology as *Aspergillus* (Ferenczy *et al.*, 1975b, 1976, 1977; Kevei and Peberdy, 1984), *Penicillium*

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(Ferenczy *et al.*, 1975b, 1976; Anne and Peberdy, 1976; Anne, 1982), *Trichoderma* (Park, 1985; Hong *et al.*, 1983, 1984; Toyama *et al.*, 1984) and *Verticillium* (Typas, 1983). Protoplast fusion, however, requires auxotrophic markers in both of the parental strains for the selection of hybrids. These mutations often affect the useful characteristics of the parents, which lower the efficiency of the process. On the other hand, such a demerit is reduced in nuclear transfer technique which was first applied for the formaiton of hybrids in the fungus, *Saccharomyces cerevisiae*, by Ferenczy and Pesti (1982) because the auxotrophic marker of the donor strain is not essential. It is also expected that hybrids between the parents with further phylogenetic distance can be obtained by the transmission of nuclei.

Previously, we employed nuclear transfer to get intraspecific hybrids in *Aspergillus nidulans*, and showed that this technique is more effective than protoplast fusion in intraspecific hybrid formation (Yang *et al.*, 1989). In the present study, we constructed interspecific hybrids between *Aspergillus* spp., *A. awamori*, *A. usamii* and *A. oryzae*, by nuclear transfer, and some characteristics of the hybrids were analyzed.

MATERIALS AND METHODS

Strains and media

The strains used in this study were *A. awamori* CM21 (Trp$^-$), *A. usamii* CM311 (Nic$^-$), CM312 (Lys$^-$), CM313 (Leu$^-$), and *A. oryzae* CM611 (Met$^-$). Strains CM311 (Nic$^-$), CM312 (Lys$^-$) and CM313 (Leu$^-$) were derived from a prototroph strain *A. usamii* CM31, and CM611 was from a prototroph strain *A. oryzae* CM61 by UV mutagenesis.

The complete and minimal media for the cultivation of these strains were prepared by the method of Harsanyi *et al.* (1977). For the regeneration of protoplasts, the minimal and complete regeneration media containing osmotic stabilizer (0.6 M KCl) were employed. For the induction of extracellular cellulase, xylanase and amylase, the strains were cultivated in a liquid minimal medium (Harsanyi *et al.*, 1977) containing 2% wheat bran in stead of dextrose.

Protoplast formation

Washed mycelia of the strains were treated with 0.5% Novozym 234 (Novo) in the osmotic stabilizer (700 mM KCl, 200 mM phosphate buffer, pH 5.8) for 2 hrs at 37℃. The protoplasts were separated from the mycelia by filtration through a sintered glass filter (porosity, 40-60 μm), and concentrated by centrifugation (700×g, 15 min).

Protoplast fusion

About 1×10^7 protoplasts from each auxotrophs were mixed, pelleted by centrifugation (1,200×g, 10 min), and resuspended in 1 m*l* of 30% PEG (M.W. 6,000) solution containing 10 mM $CaCl_2$ and 50 mM glycine. After incubation for 10 min at 30℃, the suspension was plated on the minimal and complete regeneration media containing osmotic stabilizer, and incubated for 5-8 days at 37℃.

Isolation of nuclei

The nuclei were prepared according to the method of Ferenczy and Pesti (1982). The protoplasts prepared from donor strains were suspended in 1 m*l* of SMC(0.3 M sucrose, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) solution. After 10 min incubation at 40℃, the protoplasts were homogenized, and the suspension was centrifuged (3,000×g, 10 min). The pellet was resuspended in 2 m*l* of SMC containing 0.6 M of sucrose instead of 0.3 M, and layered onto a sucrose discontinuous gradient (2.0 M, 1.5 m*l*; 1.8 M, 1.0 m*l*; 1.5 M, 1.0 m*l*; 1.2 M, 1.5 m*l*). The nucelar preparation was centrifuged at 90,000×g for 60 min. The nuclear pellet was resuspended in 1 m*l* of osmotic stabilizer, and used in the transfer process.

Transfer of nuclei

For the transmission of nuclei, a sandwich method (Ferenczy and Pesti, 1982) with some modification was adopted. The suspensions of recipient protoplasts (1.5×10^7) and donor nuclei (1.5×10^7 protoplast equivalent) were mixed in a tube, centrifuged at 1,200×g for 10 min. The supernatant was removed, and the suspension of recipient protoplasts was layered onto the precipitate. After the tube was centrifuged (1,200×g, 10 min), the pellet was resuspended in 1 m*l* of 30% PEG solution containing 10 mM $CaCl_2$ and 50 mM glycine. The suspension was diluted with the osmotic stabilizer, and plated on the complete and minimal regeneration media.

Enzyme assay

The conidia of parental and hybrid strains were cultivated in the inducing medium, and the culture filtrates were used as crude enzyme preparations.

The substrates for carboxymethylcellulase,

xylanase, and amylase were carboxymethylcellulose, xylan and starch, respectively. The reaction mixture containing 0.9 m*l* of 0.5% substrate solution in 50 mM acetate buffer (pH 5.0) and 0.1 m*l* of crude enzyme (or its diluent) was incubated at 37℃ for 15 min, and the amount of reducing sugar was measured by the method of Somogyi (1952). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol/min of reducing sugar as glucose under the conditions of the assay.

The activities of β-glucosidase and β-xylosidase were assayed by measuring the amount of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-xyloside, respectively. The reaction mixture was composed of 0.9 m*l* of 1 mM substrate solution in 50 mM acetate buffer (pH 5.0) and 0.1 m*l* of crude enzyme (or its diluent). After incubation at 40℃ for 30 min, 2 m*l* of 1 M sodium carbonate solution was added. The mixture was then diluted with 10 m*l* of water, and A_{420} was measured. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol/min of *p*-nitrophenol under the conditions of the assay.

Isoelectric focusing, electrophoresis and activity staining

Non-dissociating polyacrylamide gel electrophoresis was performed according to the method of Davis (1974) with a slight modification. Isoelectric focusing was carried out with Phast system (Pharmacia LKB). For the activity staining of carboxymethylcellulase, xylanase and amylase, an agar replica gel containing corresponding substrate was laid on the polyacrylamide gel slab, and incubated at 50℃ for an appropriate time. After the reaction was stopped by cooling the replica gel at 4℃, the gel was stained with Congo red for CMCase and xylanase and with iodine for amylase. For the activity staining of β-glucosidase and β-xylosidase, the IEF gel was laid on a filter paper soaked with 4-methylumbelliferyl-β-D-glucoside or 4-methylumbelliferyl-β-D-xyloside, incubated at 50℃ for 10 min, and observed fluorescence under UV light (350 nm).

Scanning and transmission electron microscopy

The mixture of the protoplasts and the nuclei was prefixed with 2.5% of glutaraldehyde, fixed with OsO_4, and washed twice with 10 mM phosphate buffer (pH 5.8). For scanning electron microscopy, the sample was dehydrated by serial treatment with 50, 60, 70, 80, 90, 95% and absolute ethanol. The sample was then dried for 24 hrs on a cover glass, coated with gold (400 Å thickness) on an aluminium rod, and observed under scanning electron microscope (ISI-SS40) at 25 KV. For transmission electron microscopy, dehydration of the fixed sample was performed in acetone series. The sample was embeded in Epon, and sectioned with ultramicrotome. The thin sections were placed on grids, stained with uranyl acetate and lead citrate, and observed under transmission electron microscope (Hitachi H-600) at 75 KV.

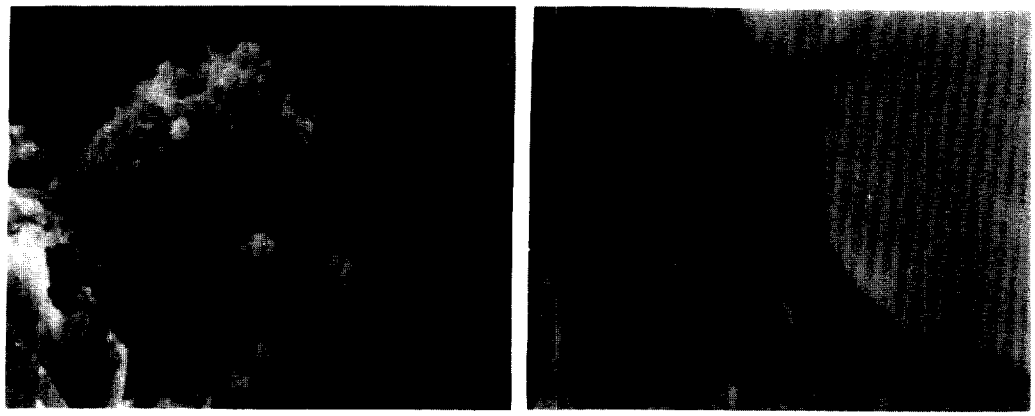
Determination of DNA content of conidia

In order to estimate the karyotype of the hybrids, the DNA content of conidia was measured. The suspension of conidia (1.0×10^8 conidia/m*l*) was centrifuged (8,000×g, 15 min), and the spore pellet was washed twice with extraction buffer containing 250 mM EDTA, 0.5% Triton X-100 and 50 mM NaCl. The pellet was then mixed with 0.2 m*l* suspension of glass bead, and the conidia were ruptured by bead beater. After the extract was centrifuged (8,000×g, 30 min, 4℃), the supernatant was harvested, mixed with 1 m*l* of absolute ethanol (−20℃), and centrifuged (8,000×g, 60 min, 4℃). The precipitated DNA was dried, and assayed by diphenylamine reaction (Giles and Myers, 1965).

Table 1. *Frequency of hybrid formation between Aspergillus spp. by nuclear transfer and protoplast fusion*

Techniques	Crosses	Frequency ($\times10^{-5}$)
Protoplast fusion	*A. usamii* CM313 (Leu$^-$)× *A. oryzae* CM611 (Met$^-$)	ND
	A. usamii CM311 (Nic$^-$)× *A. oryzae* CM611 (Met$^-$)	ND
Nuclear transfer	*A. usamii* CM313 (Leu$^-$) (N)× *A. oryzae* CM611 (Met$^-$) (P)	4
	A. usamii CM311 (Nic$^-$) (N)× *A. oryzae* CM611 (Met$^-$) (P)	5
	A. usamii CM313 (Lys$^-$) (N)× *A. oryzae* CM611 (Met$^-$) (P)	43
	A. oryzae CM611 (Met$^-$) (N)× *A. usamii* CM313 (Leu$^-$) (P)	2
	A. awamori CM21 (Trp$^-$) (N)× *A. usamii* CM313 (Leu$^-$) (P)	66
	A. usamii CM311 (Nic$^-$) (N)× *A. usamii* (M313 (Leu$^-$) (P)	170

ND: Not detected, N: Nuclei donor, P: Recipient protoplast.



(A) (B)

Fig. 1. *Electron microscopic observation of the process of nuclear transfer.*
A, A scanning electron micrograph of nuclei attached to a protoplast during the process of nuclear transfer (Mag.: 8,000 ×); B, A transmission electron micrograph of nuclei supposed to be trapped between two aggregated protoplasts and acquire access into the protoplasts during the process of cell membrane fusion (Mag.: 40,000 ×). P, Protoplast; N, Nucleus; M, Mitochondrion; PM, Plasma membrane; LV, Large vacuole.

Table 2. *Extracellular enzyme activities of hybrids obtained by interspecific nuclear tranfer between Aspergillus spp.*

Strains	CMCase (units/ml)	Xylanase (units/ml)	Amylase (units/ml)	β-glucosidase (units/ml)	β-xylosidase (units/ml)
A. awamori CM21 (Trp⁻)	0.61	16.54	9.29	2.66	2.64
A. usamii CM313 (Leu⁻)	0.50	12.93	2.61	2.60	2.65
A. oryzae CM611 (Met⁻)	0.14	10.15	39.43	1.21	0.00
		A. awamori CM21(N)×*A. usamii* CM313(P)			
HAU-1	0.66	34.99	10.38	2.67	2.66
HAU-2	0.60	27.64	7.99	2.65	2.67
HAU-3	0.61	26.08	6.80	2.61	2.66
HAU-4	0.64	21.08	9.32	2.60	2.73
		A. oryzae CM611(N)×*A. usamii* CM313(P)			
HOU-1	0.79	21.57	13.66	5.30	5.30
HOU-2	0.72	25.16	86.29	5.07	0.45
HOU-3	0.64	22.52	89.57	5.09	0.53
		A. usamii CM313(N)×*A. oryzae* CM611(P)			
HUO-1	0.61	22.03	90.69	5.24	0.55
HUO-2	0.63	28.88	78.50	5.03	4.00
HUO-3	0.63	24.20	79.62	5.00	0.41

N: Nuclei donor, P: Recipient protoplast

RESULTS AND DISCUSSION

Construction of interspecific hybrids by nuclear transfer

Some hybrids formed by protoplast fusion between two different species of *Aspergillus* has been reported since late 1970s: hybrids between *A. nidulans* and *A. fumigatus* (Ferenczy *et al.*, 1977), *A. rugulosus* (Kevei and Peberdy, 1977, 1984), *A. quadrilineatus* (Kevei and Peberby, 1984), or *A.*

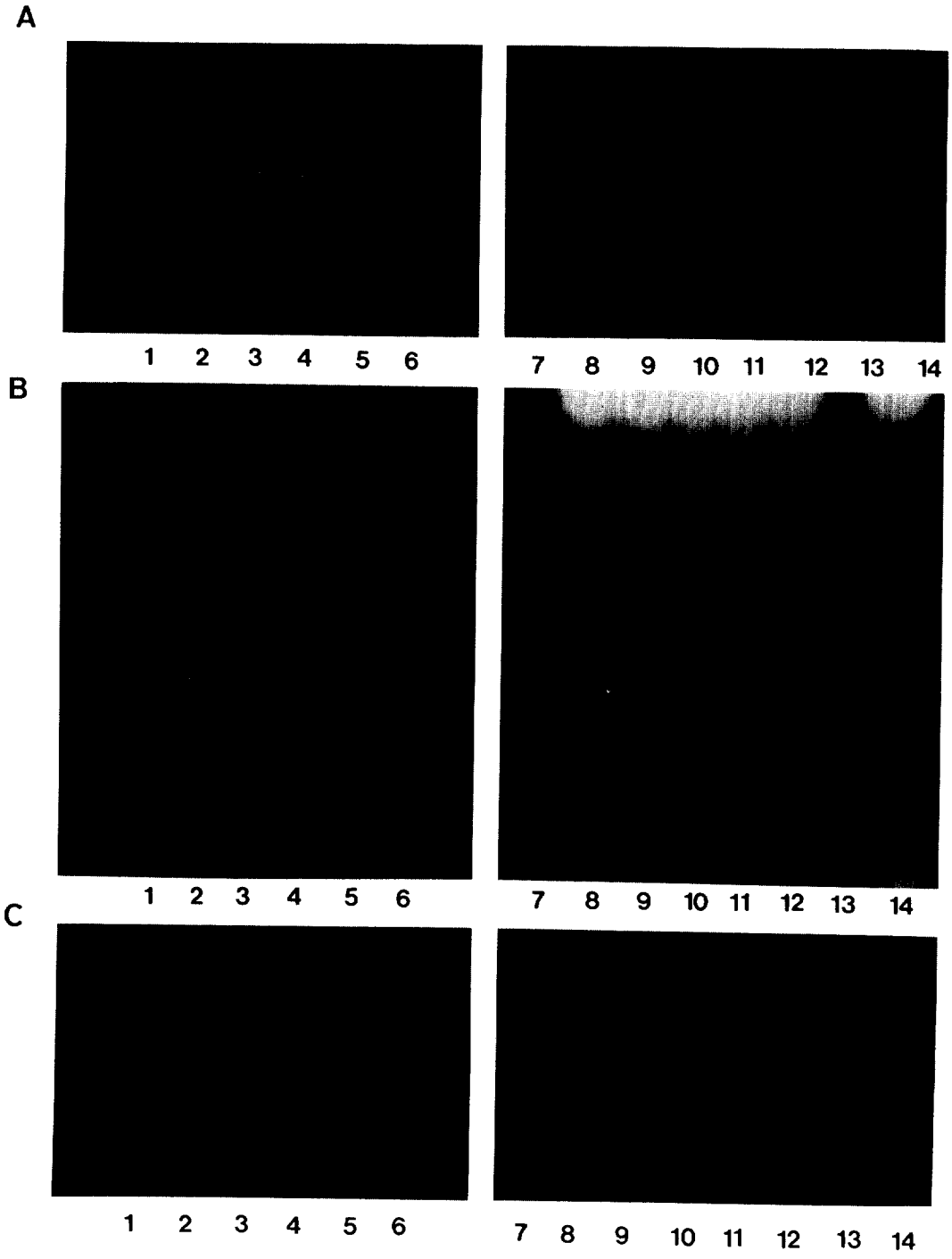


Fig. 2. *Isozyme patterns of hybrids obtained by interspecific nuclear transfer between Aspergillus spp.* Enzymes: A, CMCase; B, xylanase; C, amylase; D, β-glucosidase. Lanes: 1, HAU-1; 2, HAU-2; 3, HAU-3; 4, HAU-4; 5 and 13, *A. usamii* CM313; 6, *A. awamori* CM21; 7, HOU-1; 8, HOU-2; 9, HOU-3; 10, HUO-1; 11, HUO-2; 12, HUO-3, 14, *A. oryzae* CM611.

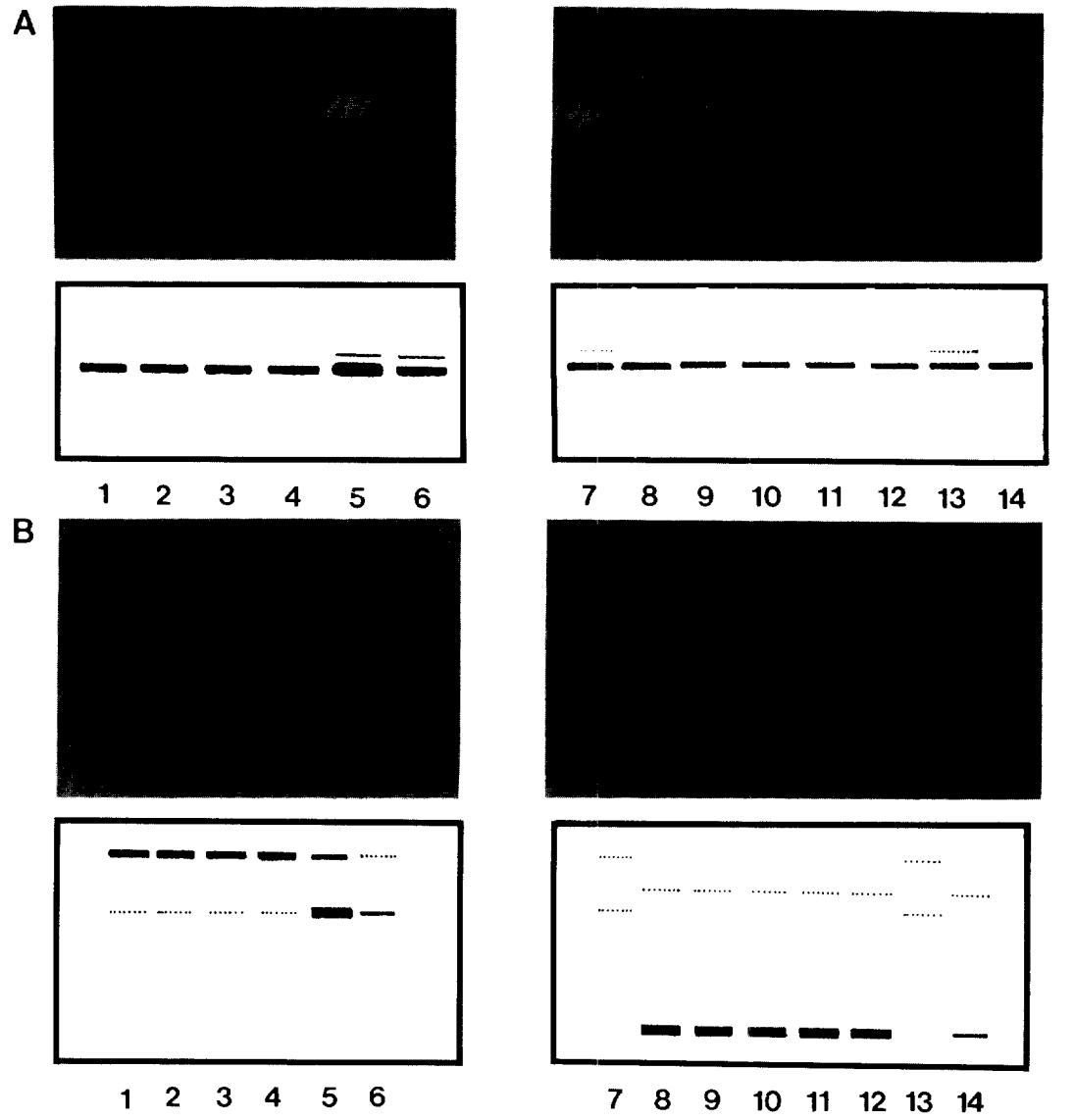


Fig. 3. *Isozyme patterns of hybrids obtained by interspecific nuclear transfer between Aspergillus spp.* Enzymes: A, β-glucosidase; B, β-xylosidase. Lanes: 1, HAU-1; 2, HAU-2; 3, HAU-3; 4, HAU-4; 5 and 13, *A. usamii* CM313; 6, *A. awamori* CM21; 7, HOU-1; 8, HOU-2; 9, HOU-3; 10, HUO-1; 11, HUO-2; 12, HUO-3, 14, *A. oryzae* CM611.

violaceus (Kevei and Peberdy, 1984). The efficiency of the hybrid formation ranged from 0.001% to 4.43% (Bradshaw *et al.*, 1983; Kevei and Peberdy, 1984). Liang *et al.* (1987) reported that the frequency of hybrid formation between two phylogenetically distant species of *Aspergillus*, *A. niger* and *A. oryzae*, was as low as $10^{-6}-10^{-5}$. These results suggest that the efficiency of hybrid construction by protoplast fusion is largely depended on phylogenetic relationship, and that protoplast fusion between the parents with large phylogenetic distance is not very efficient.

In this present work, we constructed interspecific hybrids between *Aspergillus* spp., *A. awamori*, *A. usamii* and *A. oryzae*, by nuclear tranfer. As shwon in Table 1, the frequency of interspecific hybrid formation by nuclear transfer was $2\times10^{-5}-7\times10^{-4}$, and the intraspecific hybrids were

formed at the frequency of 1.7×10^{-3}. On the other hand, interspecific hybrids were not formed by protoplast fusion. This result shows that nuclear transfer method is very useful for the construction of interspecific hybrids, and that the efficiency of hybrid formation by nuclear transfer is also affected by phylogenetic relationship as in the case of protoplast fusion.

Electron microscopic observation of nuclear transfer process

The process of nuclear transfer was followed by scanning and transmission electron microscopy. There was no evidence either for direct fusion of nuclear and protoplast membrane, or for endocytosis of nuclei into protoplasts (Fig. 1A). Fig. 1B supports the idea that, on the action of PEG-Ca^{2+} system, nuclei are trapped between two or more aggregated protoplasts, and acquire access into the protoplasts during the process of cell membrane fusion (Ferenczy and Pesti, 1982).

Analysis of interspecific hybrids

In order to screen the hybrid strains with improved degrading activity on cellulosic materials, carboxymethylcellulase, xylanase, β-glucosidase, β-xylosidase and amylase activities of interspecific hybrids were assayed, and the hybrids with increased enzyme activities are listed in Table 2. The HAU strains showed two to the three fold increase in xylanase activities when compared with their parental strains. In the cases of HOU-2, HOU-3, HUO-1 and HUO-3, activities of xylanase, amylase and β-glucosidase increased by the factor of two or more, and carboxymethylcellulase by 1.2-1.6. HOU-1 strain showed increased activity of β-xylosidase as well as carboxymethylcellulase, xylanase, amylase and β-glucosidase, and decreased activity of amylase. Especially, HUO-2 seems to be superior to the other hybrid strains in that it showed increased activities of all the enzyme components assayed. These results suggest that nuclear transfer technique can be utilized for the improvement of fungal strains.

The isozyme patterns of the hyrids were analyzed by polyacrylamide gel electrophoresis and IEF followed by activity staining. As shown in Fig. 2A and 2B, the isozyme patterns of carboxymethylcellulase and xylanase of HAU strains (lanes 1-4) were identical to those of one or both of the parental strains (lanes 5 and 6). In the cases of HOU and HUO strains (Fig. 2A and 2B), some change either in band pattern or intensity of carboxymethylcellulase and xylanase was observed. HOU-1 showed increased band intensity (lane 7), though its isozyme pattern appear to be identical to that of the recipient, *A. usamii* CM313. On the other hand, the zymograms of the remaining HOU and HUO strains (lanes 8-12) showed marked contrast to those of parents (lanes 13 and 14) in their isozyme patterns as well as band intensities. In the isozyme patterns of amylase (Fig. 2C), five of the HOU and HUO strains (lanes 8-12) showed increased band intensities. The zymograms in Fig. 3 indicate that all the hybrid strains have isozyme patterns of β-glucosidase and β-xylosidase almost identical to either of the two parents. These results suggest that the increased activities observed in the hybrids are not due to the combination of all the isozyme components of two parents, but probably due to the increase of some components of the enzyme system.

Table 3. *Conidial size and DNA content of hybrids obtained by interspecific nuclear transfer between Aspergillus spp.*

Strains	Mean conidia size (μm)	DNA content/ 10^8 conidia (μg)
A. usamii CM313 (Leu^-)	6.1±0.7	13.3
A. awamori CM21 (Trp^-)	4.5±0.5	7.3
A. oryzae CM611 (Met^-)	6.0±0.5	13.1
HAU-1	4.9±0.5	10.5
HAU-2	5.8±0.5	17.0
HAU-3	6.0±0.5	19.0
HAU-4	6.1±0.5	33.2
HOU-2	6.2±0.5	14.7
HOU-3	5.5±0.5	11.7
HUO-2	5.7±0.5	8.3
HUO-3	6.5±0.7	31.3

The karyotypes of the hybrids were estimated by measuring the DNA contents and the sizes of conidia. As shown in Table 3, two of the eight hybrid strains tested, HAU-4 and HUO-3, have 2.5-fold more DNA than the parental strains. The conidia size of the eight strains seem to remain the same. Assuming that the karyotypes of the parents are haploid, the karyotypes of HAU-4 and HUO-3 seem to be aneuploid near to diploid or triploid, and the remaining six hybrid strains to be aneuploid near to haploid.

In conclusion, it is strongly supported by the results of this study that the nuclear transfer technique is much more efficient in the formation

of interspecific hybrids than protoplast fusion. and is very useful for the improvement of *Aspergillus* strains.

적 요

섬유질 분해능이 향상된 새로운 *Aspergillus* 균주를 개발하기 위하여, *A. awamori, A. usamii, A. oryzae* 등의 균주 간의 핵전이를 통하여 종간잡종을 얻어내었다. 이들 상호간의 안정된 종간잡종은 원형질체 융합법에 의해서는 전혀 이루어지지 않은 데 반하여, 핵전이에 의한 종간잡종 형성 빈도는 $2\times10^{-5}-7\times10^{-4}$으로 나타났다. 분리된 잡종들의 효소활성도를 분석해 본 결과, 10 개의 균주에서 cellulase, xylanase, amylase, β-xylosidase, 그리고 β-glucosidase 중 일부 또는 모든 효소 활성이 모균주보다 2배 이상까지 높은 것으로 나타났다. 또한, 전기영동 및 isoelectric focusing에 의하여 일부 잡종들의 동위효소 양상이 모균주의 어느 쪽과도 일치하지 않음을 확인하였다. 포자의 크기 및 DNA 함량을 측정한 결과로 미루어 보아, 그들의 핵형은 대부분 배수체 또는 반수체에 가까운 이수체일 것으로 예측되었다. 본 연구의 결과, *Aspergillus* 속 균류에서 유연관계가 비교적 먼 종들 사이의 잡종형성을 통하여 새로운 균주를 개발하는 데 있어서 핵전이법이 원형질체 융합법보다 훨씬 더 효과적임이 밝혀졌다.

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