Gene Transfer In Edible Mushrooms

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Abstract: Distinct methods of gene transfer in fungi are protoplast fusion, uptake of foreign genetic materials and transformation. The protoplast fusion products between compatible strains, *Pleurotus ostreatus* and *P. florida,* formed heterokaryons,while fusants between incompatible strains such as *P. cornucopiae* +*P. florida* , *P. ostreatus* + *Ganoderma applanatum, P. florida* + *Ganoderma lucidum, and P. ostreatus* + *Flammulina velutipes* formed synkaryons that retained genes from both parents. Transfer of the isolated nuclei from *Lentinula edodes* into protoplasts of *P. florida* was induced. Restriction enzyme-mediated integration was used to transform uracil auxotrophs of *P. ostreatus* to prototrophy. Two of nine uracil auxotrophs obtained were transformed to prototrophy using plasmid pTRura 3-2 that contains the orotidine monophosphate decarboxylase (*ura3*) gene from *Trichoderma reesei*. This review will discuss these aspects.

Key words: Edible mushrooms, gene transfer, genetic recombination, protoplast fusion, synkaryons, transformation,

1 Introduction

The conventional breeding system in heterothallic mushrooms is based on the fertility by hyphal anastomosis. Intraspecies hyphal fusion is possible in most mushroom species. This conventional method is very useful for the development of commercial strains. Gene transfer using protoplasts has been developed to break down the barrier of gene exchange imposed by conventional breeding systems. Distinct methods of gene transfer in fungi are protoplast fusion, uptake of foreign genetic materials and transformation. The development of gene transfer systems would be useful in understanding the mechanism of genetic recombination and for strain improvement. Gene transfer as a genetic process has been well established in fungi.[1-9] This review will discuss some fundamental aspects associated with recent advances in edible mushrooms.

2 Materials and Methods

2.1 Protoplast fusion

The interspecies and interorder somatic hybrids in *Pleurotus* and *Ganoderma* were obtained by protoplast fusion and the selection of fused protoplasts as previously described.[10] (DNA isolation, amplification, and data analysis were as previously described).[11]

2.2 Transfer of isolated nuclei into protoplasts

The intergeneric nuclear transfer products of *Pleurotus florida* and *L. edodes* were obtained using protoplasts, and the selection of synkaryon, and genetic character identification as previously described.[12]

2.3 Transformation

The transformants of *P. ostreatus* were obtained using uracil auxotrophs, and the isolation and characterization were as previously described.[13]

3 Results and Discussion

3.1 Protoplast fusion

3.1.1 Development of fruiting bodies from protoplast fusion products

The protoplast fusion of *Pleurotus* and *Ganorderma* between inter-compatible and inter-incompatible species has been studied by the RDA Mushroom Research Group.[6, 10, 11, 14, 15] The somatic hybrids between compatible strains produced fruiting bodies rapidly and abundantly. Fruiting bodies of the heterokaryons showed intermediate or mixed characters between parents. Fruit body yield indices of somatic hybrids ranged between 27-155, while those of their parents, *P. ostreatus* 2018 and *P. florida* 2016 were 100 and 138, respectively.[6, 16]
Fusion products between incompatible species were derived from auxotrophic mutants of *Pleurotus* and *Ganoderma* or *Flammulina* after protoplast fusion. The interspecific somatic hybrids between incompatible pairs of strains did not form clamp connections. Out of 377 synkaryons, 35 somatic hybrids between incompatible species induced fruiting bodies (Table 1). The phenotypes of fruiting bodies in synkaryons were similar to either one of the fusion partners. Only some genetic characters including fruiting body morphology, and pileus colour were more or less different from parental species. All of the basidiocarps of synkaryons produced clamp connections except the combination of *P. cornucopiae* and *P. florida*. The amount of basidiospores from these two strains was small or even zero. None of these clampless primordia could produce mature basidiocarps. A certain part of hyphae of the clamped primordia in sawdust medium also formed clamp connections. When small tissues of the stipe from primordia or basidiocarp were cultured on CM plates, mycelial colonies grew more vigorously than those of the initial fusants and then produced clamp connections. Basidiocarp characters were similar to either one of the fusion partners except compatible fusion products between closely related species.[14, 15, 17-21]
When two different protoplasts were fused, the fused cell first produced a heterokaryon that contained both nuclei and both cytoplasms. However, most did not keep both nuclei. Eventually the two nuclei fused to form a mononuclear hybrid known as a synkaryon.[22] The cytoplasms (mitochondria) also did not remain together;

Table 1. Frequency of fertile somatic hybrids by protoplast fusion in inter-compatible and inter-incompatible species (Y.B.Yoo unpublished)

Fusion combination	No. examined	No. fruiting (%)
Inter-compatible species		
P. ostreatus* + *P. florida	**78**	**78 (100)**
Inter-Incompatible species		
Pleurotus citrinopileatus + *P. florida*	73	13 (17.8)
P. ostreatus + *P. sajor-caju*	70	7 (10.0)
P. ostreatus + *G. applanatum*	36	5 (13.9)
P. florida + *G. lucidum*	46	8 (17.4)
P. ostreatus + *G. lucidum*	60	0 (0)
P. ostreatus + *F. velutipes*	92	2 (2.17)
Total	377	35 (9.3)

eventually only one species predominated although some mitochondria fused together.[19, 23-25] After fusion, the nuclear and mitochondrial genomes reassorted and recombined, resulting in a wide array of gene recombination that cannot be obtained through hyphal anastomosis. The interspecific and intergeneric fusants between vegetatively incompatible species in *Pleurotus* and yeast were revealed to be uninucleate in a cell.[26, 27] Intergeneric somatic hybrids were not real heterokaryons because of asynchronous fruiting body development of the two partners.[17] We can obtain several kinds of somatic hybrids by protoplast fusion such as diploid, heterokaryon (inter-compatible), synkaryon, and heterokaryotic synkaryon (inter-incompatible strains).

The major species of *Pleurotus* are all bifactorial heterothallic. Single basidiospore isolates from fruiting bodies are homokaryotic and self-sterile. However, homokaryotic fruiting has been reported in some species of *Pleurotus*. We found that homokaryons derived from one strain (WT-2029) could develop fruiting bodies of three different types. The first group did not have only mature or sporulate fruiting bodies but also clamp connections, which initial isolate also did not present clamp connections (abortive homokaryotic fruiting, AHF). The second group had developed fruiting bodies with clamp connections even though initial homokaryotic colonies did not form clamp connections (pseudo-homokaryotic fruiting, PHF). The mycelial colonies derived from AHF and PHF by tissue culture formed clamp connections, while mycelial colonies of AHF lacked them (Y.B. Yoo unpublished).

However, monokaryotic auxotrophs of *Pleurotus* and *Ganoderma* in these experiments were self-sterile. The clampless fusant did not produce fruiting bodies on complete agar medium or complete liquid medium in flasks. Clamp connections of initial fusion products did not form in the phase of vegetative mycelial growth on sawdust substrates. Light and low temperature were main factors to initiate the emergence of clamp connections in hyphae from clampless initial fusion colonies. When clamped mycelia were grown completely, fruiting bodies developed on sawdust substrates. These results indicate that formation of clamp connections and development of fruit bodies is influenced by some factors such as light, temperature, nutrition, mycelial age, and physical state of culture media. Fruiting body inducing genes were silent in vegetative mycelial growth but became active when aerial hyphae were exposed to light at low temperature.

Monokaryotic fruiting has been observed in species of six genera of the Poriales and 12 genera of the Agaricales.[28] Esser suggested that there are at least two genes that control the potential for monokaryotic fruiting. In the presence of allele *fi* + only fruiting initiation occurred; the additional presence of allele *fb* + led to production of normal fruiting bodies.[29, 30] There are several factors related to the mechanism of clamp connection formation and fruiting body development of synkaryons between incompatible species. The major ones may be associated with self-fertility and mating type switching as reported earlier.[31, 32]

Development of fruit bodies in synkaryons depends on self-fertility of initial wild fusion partners. We found that synkaryons could develop fruit bodies of two different types. The first group developed fruit bodies with clamp connections. The second group did not have mature fruit bodies. There are several factors related to the mechanism of fruit body development in synkaryons by protoplast fusion. The major factors may be associated with self-fertility and mating type switching (Figure 1). Gene expression during emergence of clamp connections in synkaryons may be linked to the mating type switching. This is related to the function of heterokaryotization of synkaryons without transfer of mating type genes or nuclei, and eventually leads to the differentiation and development of mature fruiting bodies. These results indicate that basidioma development and mating system pattern of somatic hybrids between incompatible species were similar to those of self-fertile homokaryons of wild type *P. ostreatus*.

3.1.2 Genetic recombination

Somatic hybrids of inter-compatible and inter-incompatible strains were analysed for the segregation and recombination of progenies by random spore analysis. The genetic markers were shown to segregate and recombine in the first generation of monospore isolate. Progenies of the somatic hybrids can be classified into four

genotypes: auxotrophs of one parental type, auxotrophs of the other parental type, prototrophs, and auxotrophic recombinants. In all somatic hybrids, however, prototrophic recombinants were recovered in large numbers against auxotrophic characters. Several somatic hybrids did not segregate with one parental genetic marker. Some were shown to be segregated into non-parental auxotrophs in the progenies of *P. citrinopileatus* + *P. florida* and *P. ostreatus* + *G. applanatum* pairings. In the somatic hybrids of dikaryotic *P. florida* and monokaryotic *P. citrinopileatus* strains, all the genetic characters from parents were segregated and recombined in the progenies. Comparatively large numbers of prototrophic recombinants were recovered from most types of fusant. This phenomenon may be related to the amphithallism and germination frequency of basidiospores derived from particular genotypes. Similar results have been obtained in the heterokaryon of basidiomycete fungi.[27, 33-36]

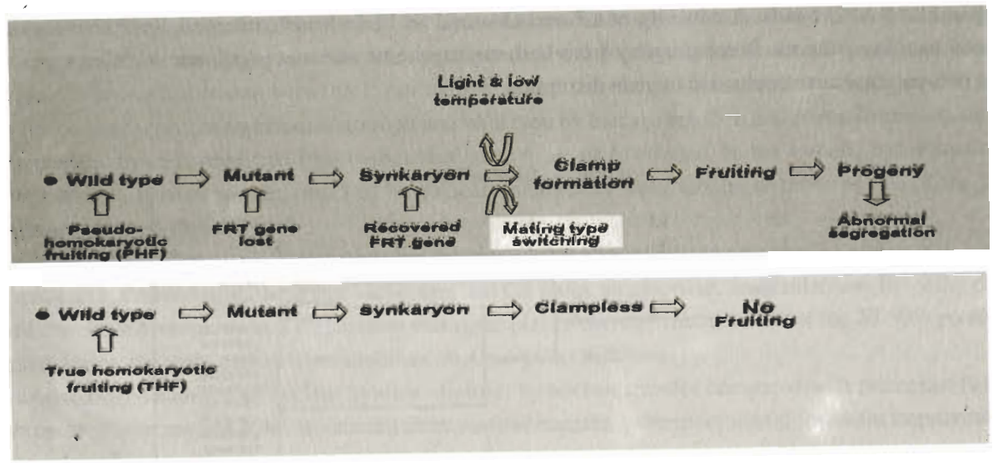

Figure 1. Comparision of basidioma development of fertile and sterile synkaryons in *Pleurotus* (Y.B.Yoo unpublished)

For heterokaryotic synkaryons, somatic hybrids showed an unequal distribution of different nuclei in the hyphae even though they are mononucleate in a cell. Because most genetic markers of the two fusion partners are segregated and recombined in the progeny, a more frequent distribution of one nuclear type over the other will lead to the phenotype. Characteristics of only one parental phenotype after genome reassortment can be explained by an unequal contribution of both nuclei to the fusion products. If heterokaryons occur in the incompatible pairings and one of the nuclear combinations exhibits a dominant, only one parental phenotype will be detected in the heterokaryotic synkaryon hyphae.

3.1.3 Molecular genetic analysis with RAPD markers

Genetic variations within or among inter-compatible and inter-incompatible somatic hybrids of Pleurotus were assessed using RAPD markers. PCR amplification of DNA from 15 strains of *P. ostreatus* + *P. florida*(OF), 18 strains of *P. citrinopileatus* + *P. florida*(CF), 15 strains of *P. ostreatus* + *G. applanatum*(OA), and 21 strains of *P. florida* + *G. lucidum*(FL) was carried out using 39 primers. A specific DNA band pattern resulted for somatic hybrids with each amplimer. To determine genetic relationships among parental strains and somatic hybrids, a dendrogram was generated from the similarity matrix using UPGMA cluster analysis (Figure 2).

RAPD-PCR patterns of 11 inter-compatible heterokaryons in pairing of *P. ostreatus* and *P. florida* by hyphal anastomosis and protoplast fusion were compared with the parental strains. No polymorphism was found among 11 heterokaryons, which did not contain non-parental RAPD bands. The inter-compatible somatic hybrids were

classified into three major groups using the 75 RAPD bands obtained. To assess the genetic variability of inter-incompatible somatic hybrids, RAPD analysis was performed each fusion combination. RAPD profiles of inter-incompatible somatic hybrids revealed a high polymorphism of DNA fragments compared with inter-compatible species.

We did not detect a change in the major RAPD banding patterns of microgenome insertion type when compared with the recipient (dominant) partner. In this case, most of the genome of one fusion partner is lost (donor or recessive). However, some genetic materials remain in the chromosomes of the other fusion partner. Despite the fact that the donor genetic material consists of full-size chromosomes from nuclei, most somatic hybrids in CF and OA pairings receive small subchromosome fragments. In most cases, the genetic similarity between the somatic hybrids and the recipient parent was over 80%. However, most genetic markers of two fusion partners were segregated in the progenies. Macrogenome insertion types were recognized by the appearance of intense non-parental RAPD bands. A minority of CF and OA, and all FL hybrids contained detectable major non-parental banding patterns. Some progeny from both microgenome and macrogenome insertion types segregated non-parental auxotrophs due to gene disruption.[19, 21]

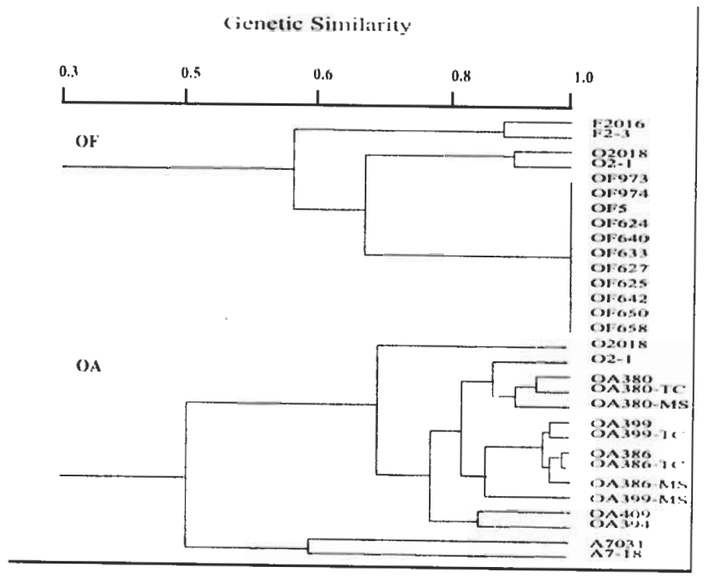

Figure 2. Relationships of parentals and somatic hybrids in pairings of (OF) *Pleurotus ostreatus* + *P. florida*, and (OA) *P. ostreatus* + *G. applanatum*
The dendrogram was generated from genetic similarity coefficients obtained from 75, and 127 DNA bands, respectively, using UPGMA cluster analysis.[11]

3.2 Transfer of isolated nuclei into protoplasts

The transfer of isolated nuclei from protoplasts or mycelia into protoplasts has been studied in mushrooms.[12, 37-39] The intergeneric nuclear transfer products arising from donor nuclei of *L. edodes* (wild) and recipient protoplasts of *P. florida* (ribo) were nuclear hybrids, synkaryons, and reconstitute cells. Nuclear hybrids were more vigorously growing abundant mycelia and stable colonies on CM. When cultured in the presence of benomyl (1-100mg per ml) the mycelial colonies could be broken. Nuclear hybrids secreted a dark blue pigment on CM. They neither formed clamp connections nor produced fruiting bodies (Table 2). These products were assumed to be a hybrid after nuclear fusion in a cell. These allodiploid or heteroploid were non-fertile. Characteristics of

these hybrids were similar to those of nuclear hybrids of higher fungi except pigment production.[18, 37] The pigmentation produced by the hybrids was proved to be melanin,[40] and was associated with tyrosine metabolism as reported earlier.[41] Segregation colonies were obtained from hybrids grown in the presence of benomyl, chloral hydrate and paraflurophenylalanine.[23, 40]

Most of the transfer products were synkaryons. The clampless colonies did not produce fruiting. Clamp connections did not form during vegetative mycelial growth on sawdust substrates. Light and low temperature were initiating factors in the development of clamped hyphae from clampless mycelial colonies. When clamped mycelia from clampless mycelial colonies were grown completely, fruiting bodies developed on sawdust substrates in glass bottles similar to results with protoplast fusion products between incompatible species.

Intergeneric synkaryons showed various mixed colours of parental species. The genetic character of *P. florida* was predominant. These results indicate that, after fusion, the nuclear genomes reassort in a cell. When the protoplasts and karyoplasts first fused, a heterokaryon was produced which contained both nuclei. They did not stay together and eventually the mononuclear synkaryon cell was produced after fusion of the nuclear genomes. Intergeneric protoplast fusion between *P. cornucopie* and *L. edodes* has been reported by Ogawa.[20] In this case, fusion products obtained from auxotroph and wild type by barrage reaction and clamp formation analysis. These somatic hybrids produced fruit bodies similar to those of *L. edodes*. In the somatic hybridization between distinctly related species, most of basidiocarp characters were similar to those of one of the fusion partners as reported earlier.[6, 17, 19]

The reconstituted cell with clamp connections developed into the fruit body was similar to that of *L. edodes*. This product is derived from the donor karyoplast and the recipient cytoplast. In filamentous fungi the proportion of enucleate protoplasts in a preparation was quite high. Peberdy[42] suggested that the 20-50% protoplasts released during the early period were enucleate in *Aspergillus nidulans*.

We obtained large numbers of nuclear hybrids (diploid) by nuclear transfer compared with protoplast fusion in mushrooms. These nuclear hybrids, derived from nuclear transfer, were more useful for strain improvement in medicinal mushrooms.

Table 2. Characteristics of intergeneric transfer products following the uptake of isolated nuclei of *L. edodes* by protoplasts of *P. florida* [12]

Species[1]	Genetic background	Mycelial colony		Fruiting		No. of isolates
		Growth[2]	Clamp[3]	Type	Clamp[4]	
Transfer	Reconstituted cell	M	+	*L. edodes*	+	1
Products	Synkaryon 1	M	-	*P. florida*	+	11
	Synkaryon 2	M	-	Primordial	-	2
	Synkaryon 3	M	-	non-fertile		2
	Nuclear hybrid	F	-	non- fertile		2
L. edodes-w-d	Donor	M	+	*L. edodes*	+	
P.florida-rib-m	Recipient	M	-	non-fertile		

[1] W(wild), rib(riboflavine), d(dikaryon), m(monokaryon), [2] M(moderate growing), F(fast growing), [3,4] +(Present clamp connection), -(Absent clamp connection)

3.3 Transformation

3.3.1 Efficient transformation of *P. ostreatus* using REMI method

Transformation in fungi have been achieved by various methods, e.g., protoplast + polyethylene glycol,[43, 44] lithium acetate,[45, 46] liposome,[47] electroporation,[48, 49] restriction enzyme-mediated integration,[50] particle bom

bardment[51] and *Agrobacterium*.[52-55]

The restriction enzyme-mediated integration (REMI) method was first described in transformation of *Saccharomyces cerevisiae*.[50] This technique has been employed for the disruption, tagging, and identification of genes in *Dictyostelium discoideum*,[56]*Cochliobolus heterostrophus*,[57] *Ustilago maydis*,[58] *A. nidulans*[59] and *Coprinus cinereus*.[60] REMI was used to transform uracil auxotrophs of *P. ostreatus* to prototrophy. Plasmid pTRura3-2 has a unique restriction site for *Bam*HI and *Hind*III. The effects of enzyme amounts on transformation efficiency were determined using these restriction enzymes when 1 μg of circular pTRura3-2 DNA was added into 1×10^7 protoplasts. When protoplasts of *P. ostreatus* were treated with a reaction mixture containing 10 units of *Bam*H1, efficiency was increased 14.2 times compared with that of the conventional PEG method. Uracil auxotrophs of *P. ostreatus* were isolated using the selectable marker, resistant to 5'-fluoro-orotic acid (5'-FOA).[61]

3.3.2 Transformation of uracil auxotrophs in *P. ostreatus*

There are two types of vector for transformation in fungi. The naked DNA donor can established itself as an autonomously replicating sequence (ARS) or integrate into the host chromosome after entry into the recipient cell. DNA-mediated transformation of *Pleurotus* mushrooms have been studied in our RDA Mushroom Research Group.[13, 61-65] In order to obtain uracil auxotrophs from *P. ostreatus* strain ASI2029, 10^8 basidiospores were mutated and plated on 5'-FOA media. Among the 5'-FOA resistant colonies that emerged, 125 were selected for further characterization. Nine *P. ostreatus* uracil auxotrophs were isolated from these colonies. Since no *ura* genes have been cloned from *P. ostreatus, ura* genes from *T. reesei* were used for transformation to see whether these vectors could complement the uracil mutants of *P. ostreatus*. Transformation of five of the nine *P. ostreatus* uracil auxotrophs was attempted using the PEG/$CaCl_2$ method and both the pTRura 3-2 and pTRura 5-3 vectors. From these five auxotrophs, two strains were transformed to prototrophs using the pTRura 3-2 vector, while no prototrophs were recovered using the pTRura 5-3 vector. One of the two strains transformed with pTRura 3-2 was used as the host for further experiments.

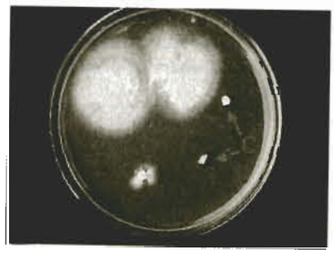

Figure 3. Transformants of *P. ostreatus* growing on MM

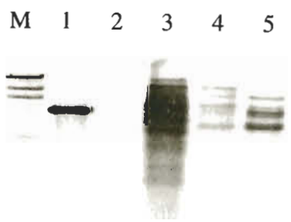

Figure 4. Southern hybridization of *Eco*RI-digested genomic DNA extracted from the uracil mutant strain *P. ostreatus* ASI2029-8, and three recombinant strains transformed with pTRura 3-2
M: DNA size marker (DIG-labelled λ/Hind III), lane 1: positive control (EcoRI-digested pTRura 3-2), lane 2: negative control (untransformed ASI2029-8), lane 3, 4, 5: T1, T2, T3 transformants (Modified from [13])

The total DNA extracted from *P. ostreatus* transformants obtained in this study was digested with *Eco*RI and hybridized with DIG labelled pTRura 3-2 (Figure 3). The different Southern hybridization profiles of the three transformants indicated that the DNA was inserted into different sites in the genome. Many hybridizing bands of high intensity were detected in transformant T1, indicating a great number of insertions or tandem repeat insertions within the T1 genome. Transformants T2 and T3 showed only two major hybridizing bands, indicating one or two insertions. ASI2029-8 strain didn't show the hybridizing bands, suggesting that this gene has a low level of nucleotide homology between *P. ostreatus* and *T. reesei*. For fruiting body induction in the three *P. ostreatus* transformants, we crossed them with the compatible wild type monokaryon strain, ASI2018-247. The resulting fruiting bodies showed normal morphologies.

Table 3. The relative merits of different methods of gene transfer in mushrooms (Y.B.Yoo unpublished)

Method	Inter-incompatible species	Polygene transfer	Singlegene transfer	Direct genetic recombination	Recovered fruit body development
Hyphal anastomosis	-	+	-	-	+
Protoplast fusion	+	+	-	+	+
Nuclear transfer	+	+	-	+	+
Transformation	+	-	+	+	-

+ ; relative success

In this study, we have shown that the *ura3* gene of *T. reesei* can complement the 5'-FOA resistant mutants of *P. ostreatus*. This suggests that the system can be adapted as an efficient and rapid transformation system for basidiomycetes in general.

There are technical advantages and disadvantages to various gene transfer methods (Table 3). Protoplast fusion and nuclear transfer may be useful in crosses between compatible and incompatible species. In this way, shuffling of genes within a limited polygenic system is still possible. Most of initial wild fusion partners such as *P. ostreatus* and *P. florida* were self-fertile. However, auxotrophic mutants of them for fusion in these experiments were self-sterile. Gene transfer products derived from protoplast fusion and nuclear transfer recovered their fertility. Single gene transfer between incompatible species may be most profitably carried out using DNA-mediated transformation.

4 Conclusions

Somatic hybrids of inter-compatible species formed heterokaryons, but somatic hybrids of inter-incompatible species formed synkaryons by protoplast fusion and nucleus transfer. Development of fruit bodies in synkaryons depends on self-fertility of initial wild fusion partners. We would note that inter-incompatible fusion products produced poor fruiting bodies compared with wild parental strains. However, recombinant progeny derived from such fruiting bodies may provide many possibilities for strain improvement in edible fungi. The main purpose for the development of DNA-mediated transformation is its application in molecular biology studies. Development of gene transfer systems opens up many possibilities for mushroom production and mushroom products. Such genetic transformations or mutations using gene transfer methods could involve those affecting growth rate, fertility, sporulation, fruiting body yields, and substrate utilization efficiencies in edible and medicinal mushrooms.

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