

Current Technologies and Related Issues for Mushroom Transformation

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Abstract Mushroom transformation requires a series of experimental steps, including generation of host strains with a desirable selective marker, design of vector DNA, removal of host cell wall, introduction of foreign DNA across the cell membrane, and integration into host genomic DNA or maintenance of an autonomous vector DNA inside the host cell. This review introduces limitations and obstacles related to transformation technologies along with possible solutions. Current methods for cell wall removal and cell membrane permeabilization are summarized together with details of two popular technologies, *Agrobacterium tumefaciens*-mediated transformation and restriction enzyme-mediated integration.

Keywords *Agrobacterium*, Mushroom, Protoplast, REMI, Transformation

MUSHROOM LIFE CYCLE AND PERSPECTIVES

Mushrooms are fruiting body-forming fungi normally belonging to Ascomycota or Basidiomycota. These fungi normally grow on plant materials by propagating vegetative mycelia through elongation of hyphae. Mycelia can be either dikaryotic (N + N) or monokaryotic (N). Monokaryotic mycelia are composed of mononuclear cells, which generally appear in most of life-cycle stages of Ascomycota as well as in mycelia generated from basidiospores of Basidiomycota. Dikaryotic mycelia are formed by hyphal fusion (anastomosis) of compatible monokaryotic mycelia. Basidiomycetes spend most of their vegetative lives as dikaryotic mycelia. Mushroom fruiting bodies are masses of dikaryotic mycelia with specialized structures, called basidia (2N), which make sexual spores such as basidiospores and ascospores as a means of sexual reproduction.

From a practical point of view, fruiting bodies are often

consumed as food sources due to their unique flavors. Edible mushrooms, including *Agaricus bisporus*, *Flammulina velutipes*, *Lentinula edodes*, *Pleurotus eryngii*, and *P. ostreatus*, are readily available at any food market owing to their commercial cultivation. In addition to their nutritional consumption, mushrooms have been recently explored for new applications, including bioremediation, cell factories for foreign protein production [1, 2], value-added material sources [3, 4], and medicines [5, 6].

Mushrooms are ubiquitous organisms found in almost every ecosystem and play central roles in the recycling of organic matter. A considerable amount of literature has been published on the ecology, physiology, genetics, and biotechnology of mushrooms. Moreover, more than 100 genomes of Agaricomycotina alone have been sequenced and are openly available in a public database (<http://genome.jgi.doe.gov/programs/fungi/>). However, knowledge of the molecular cell biology of mushrooms has been restricted to only a few model mushrooms, such as *A. bisporus* and *Coprinopsis cinereus*, due to lack of molecular biological tools related to transformation. This review lists current obstacles that hinder the introduction of foreign genes into mushroom cells along with current solutions.

MAJOR OBSTACLES TO MUSHROOM TRANSFORMATION

Introduction of foreign DNA into mushroom cells should be readily accessible at any stage of the life-cycle in order to better understand mushroom biology as well as generate mushrooms with new industrial applicability. However, transformation of mushrooms is very difficult due to the following reasons.

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Connected cells with apical growth. Fungal cells are connected to form haphae, which are highly interconnected and form mycelia. For this reason, isolation of single mushroom cells is impossible. Growth (division) of filamentous fungi only occurs at the tip of mycelia, which means most cellular machineries for growth are concentrated at the apical tip of cells [7]. Moreover, cytoplasm of cells in mycelia are connected by septal pores (dolipores for Basidiomycota), through which molecules and even subcellular organelles can pass so that cells without selective markers survive [8, 9]. Use of germinating spores, finely broken mycelia with regenerated apical tips, or protoplasts can circumvent the issue of connected cells.

Thick cell wall. Fungal cells in vegetative mycelia have thick cell walls, mainly composed of chitin, β -1,3-glucan and β -1,6-glucan, and glycoproteins. *Penicillium brevicompactum* has a thickness of 149 nm [10, 11], whereas Gram-negative and Gram-positive cell walls have thicknesses of 5~10 nm and 20~80 nm, respectively [12]. Spores of fungi have even thicker walls of approximately 1 μ m [13]. At least some cell walls have to be removed to introduce foreign DNA.

Heterokaryosis. In Basidiomycota, vegetative mycelial cells normally contain two different nuclei with compatible mating types [14, 15]. Therefore, if foreign DNA is not integrated into both nuclei at the same time, which is obviously very hard to achieve, then transformant carrying the selective marker will be diluted through the sporulation process during either sexual or asexual life-cycle when there is no selective pressure. Therefore, it is desirable to use monokaryotic cells for maintenance of the transformant.

Few molecular biological tools. There are very few available molecular biological tools for mushroom transformation. Unlike yeast, few natural nuclear plasmids have been discovered in filamentous fungi while plasmids found in filamentous fungi are mostly mitochondrial plasmids [16]. Synthetic plasmids with an autonomous replicative sequence (ARS), promoter, and selective marker from various origins have been developed for many fungi in Ascomycota and some in Basidiomycota [17, 18]. Moreover, vast numbers of hosts for *Saccharomyces cerevisiae* are available with sets of auxotrophic markers (e.g., *leu2-3, 12, trp1-1, ura3-1, ade2-1, and his 3-11, 15* in W303-1A strain [19]) while few auxotrophs have been generated in mushrooms. *C. cinereus* is one of the most studied model basidiomycetes, and efforts have been made in order to generate mutant strains with selective markers, but few are available, including *trp-2* [20, 21], *met, his,* and *cystathionine* [22].

Host defense mechanism. DNA double-strand breaks (DSBs) are detrimental and therefore must be repaired. DSBs in the eukaryotic genome are mainly repaired by homologous recombination (HR), single-strand annealing,

microhomology-mediated end-joining, and non-homologous end-joining (NHEJ) [23-25]. These repair systems also play roles in the integration of foreign DNA into eukaryotic genomes mainly via HR and NHEJ. HR is the major player in yeast, and thus yeast transformation can be easily facilitated using homologous regions of the gene of interest [26]. On the contrary, filamentous fungi and higher eukaryotic organisms use NHEJ as their main repair system, such as *KU70, KU80,* and *DNL4* in *S. cerevisiae* [27]. As a result, integration of exogenous DNA for the transformation of filamentous fungi is considered to be highly difficult since it requires DSBs, which are random and relatively rare [27]. Deletion of *MUS-51* and *MUS-52*, which are homologous to *KU70* and *KU80*, respectively, or *MUS-53*, which is homologous to *DNL4* or *Lig4* in humans, highly increases the integration of exogenous DNA through HR in *Neurospora crassa* [27, 28]. This is also true for basidiomycete fungus *C. cinerea*. Disruption of *Cc.ku70* or *Cc.lig4* has been shown to cause high-frequency gene targeting in oidia and mycelia of *C. cinerea* [29]. Therefore, disruption of NHEJ-related genes in a target host is apparently one of the prerequisite steps in mushroom transformation.

Maintenance of foreign DNA inside mushroom cells. No nuclear or cytoplasmic plasmid DNA has been discovered in mushrooms so far. Maintenance of a synthetic plasmid in mushroom cells is strictly dependent on selective marker. However, maintenance of constant selection pressure is hardly achievable since mushroom cultivation can last for several months in solid medium while producing potent degradative enzymes. Moreover, cell division only occurs at the apical tip of cells. The plasmid has to be multiplied at the tip of the cells and segregate into the dividing cell. Synthetic vector containing mushroom mitochondrial ARS or mycovirus with a target gene can be developed as mushroom vectors but need further development. Direct integration of linear DNA into the host genome appears to be the only way to maintain foreign gene integration for now.

METHODS FOR MUSHROOM TRANSFORMATION

Mushroom transformation has been performed using various samples at different life cycles with various methods, including *Agrobacterium tumefaciens*-mediated transformation (ATMT), polyethylene glycol (PEG)-mediated protoplast transformation, restriction enzyme-mediated integration (REMI), electroporation, and ballistic bombardment, along with following questions and solutions:

Removal of thick mushroom cell wall. As described above, mushroom cells are surrounded by a thick cell wall with layers of chitin, β -glucan, and glycoproteins. Removal of the cell wall is a prerequisite for most of the transformation protocols. Several hydrolytic enzymes from a fungal pathogen,

Table 1. Cell wall lysing enzymes and osmotic stabilizer

Species	Lysing enzyme	Osmotic stabilizer	Reference
<i>Coprinus congregatus</i>	Novozyme 234 ^a	0.55 M Sorbitol	[30]
<i>Flammulina velutipes</i>	1.5% Cellulase, 1.5% lysing enzyme	0.75 M MgSO ₄	[31]
<i>Ganoderma lucidum</i>	Novozyme 234	0.55 M Sorbitol	[32]
<i>Lentinula edodes</i>	2.5% Cellulase, 0.1% chitinase	0.6 M Mannitol	[33-35]
		1.2 M Sorbitol	[36]
<i>Pleurotus eryngii</i>	0.25% Lysing enzyme ^b	1 M MgSO ₄	[37]
	2% Lywallzyme ^c	0.6 M Mannitol	[38]
<i>Pleurotus nebrodensis</i>	1.5% Lywallzyme	0.6 M MgSO ₄	[39]
<i>Pleurotus ostreatus</i>	2% Novozyme 234, 0.2% chitinase, 0.5% Zymolyase ^d	0.6 M Mannitol	[40, 41]
	0.5% Novozyme, 0.5% glucanase, 0.5% Glucuronidase	0.6 M Sucrose	[42]
	1% Novozyme 234	0.8 M Mannitol	[43]
	2.5% Lywallzyme	0.6 M Mannitol	[44]
<i>Trametes vesicolor</i>	Novozyme 234	0.5 M Sorbitol	[45]

^aNovozyme 234 is a multienzyme mixture, consisted of β -glucanase, cellulase, protease, chitinase, and α (1,3)-glucanase, mainly from *Trichoderma harzianum*. It is available from Sigma-Aldrich under the name of Glucanex or lysing enzyme.

^bLysing enzyme is a Sigma-Aldrich version of Novozyme 234.

^cLywallzyme appears to be a similar mixture of lytic enzymes to Novozyme 234 but from *Trichoderma longibrachiatum* [86].

^dZymolyase is a β (1,3)-glucanase produced from *Arthrobacter luteus* [46].

Trichoderma harzianum, are available as mixtures of chitinase, β -glucanase, and protease (Table 1) [30-45]. The enzyme reaction with mushroom tissue or mycelia should be conducted in the presence of a high concentration of osmotic stabilizers, including sorbitol [30, 32, 36, 45], mannitol [33-35, 38], sucrose [42], and MgSO₄ [31, 37, 39], since protoplasts and spheroplasts are sensitive to osmotic pressure. The optimal concentration of osmotic stabilizers is around 0.6 M.

Permeabilization of mushroom cell membrane. The cell membrane is the primary barrier preventing introduction of foreign DNA into the cytoplasm. Permeabilization of the cell membrane has been performed by various physicochemical methods. In *S. cerevisiae*, treatment with PEG together with lithium acetate (LiAc) and single strand DNA (ssDNA) enables high efficiency transformation of yeast cells [47]. PEG is known to increase membrane permeability through interaction with membrane lipids [48]. LiAc and ssDNA also increase the permeability of

nucleic acids, although the detailed mechanism is not fully understood [49, 50]. PEG treatment has been applied to the transformation of *Pleurotus* mushrooms using PEG3350 or PEG4000, as shown in Table 2 [38, 39, 41, 43, 44, 51-58]. Electroporation is a method for local disturbance of the cell membrane by applying electric pulses with a certain electric potential (V) and capacitance (F). Variables in this method are the intensity (ranging 5~10 kV/cm) and duration (few msec) of the electric pulse. The intensity can vary depending on the applied electric potential (V) and the width of the cuvette (0.1 or 0.2 cm). Typical settings for mushroom transformation are summarized in Table 2. Ballistic bombardment is a transformation method using helium pressure to introduce DNA-coated gold particles into cells. This method has been generally applied for the transient expression of genes in plants, and it has been seldom applied for mushroom transformation [57, 58]. *Agrobacterium tumefaciens* can make holes in the membrane of any living organism. *A. tumefaciens* produces proteins related to host interaction and gene transfer from the *virB*

Table 2. Techniques for the introduction of foreign DNA to mushroom protoplasts

	Species	Cell type	Conditions	Plasmid	Reference
PEG treatment	<i>Pleurotus eryngii</i>	Monokaryotic	PEG3350	pEPVGH	[38]
	<i>Pleurotus nebrodensis</i>	Dikaryotic	PEG4000	pBlue-GFP	[39]
	<i>Pleurotus ostreatus</i>	Dikaryotic	PEG4000 or 3350	pAN7-1, pTM1	[41, 43, 44]
Electroporation	<i>Agaricus bisporus</i>	Dikaryotic	0.45 kV, 25 mF, 200 Ω	pAN7-1, pA2H	[51]
	<i>Flammulina velutipes</i>	Dikaryotic	1.25 kV, 25 mF, 100 Ω	pFGH	[52]
		Monokaryotic	1.25 kV, 25 mF, 200 Ω	pFTXHg	[53]
	<i>Ganoderma lucidum</i>	Dikaryotic	2.5 kV, 25 mF, 400 Ω	p301-bg1	[54, 55]
	<i>Lentinula edodes</i>	Spore	6.25~12.5 kV, 25 mF, 100~800 Ω	pL-gus, pLi-gus	[56]
Ballistic bombardment	<i>Pleurotus ostreatus</i>	Gold particle: 0.6 μ m		pTRura 3-2	[57]
	<i>Lypophyllum decastes</i>	He: 650~1,300 psi Target distance (cm): 5, 8, 11, 14.5		pHHM192	[58]

operon in Ti-plasmid [59]. The *virB2* gene encodes T-pilin protein for assembly of the T-pilus, with which the bacteria attach to hosts cell and function as a channel for T-DNA transfer. Some examples are described in below.

***Agrobacterium tumefaciens*-mediated transformation.**

A. tumefaciens can infect any higher organism and transfers a DNA fragment (transfer DNA, T-DNA) from a tumor-inducing plasmid (Ti-plasmid) into the host genome via activity of *vir* gene product, which is contained in Ti-plasmid. Using this property, ATMT has been applied to the transformation of a variety of filamentous fungi, including *Aspergillus*, *Fusarium*, and *Trichoderma* [60-63]. Spores, mycelia, protoplasts, and tissues of fruiting bodies from basidiomycete fungi also have been subjected to ATMT (Table 3) [61, 64-81]. Successful ATMT relies on several

factors. *A. tumefaciens* strains were shown to differentially affect the transformation efficiency of tomato and the copy numbers of genes integrated in the tomato genome [82]. Although the AGL-1 strain shows the best performance for tomato, the effect of the strain can vary depending on the organism and transformation conditions (Table 3). The cultivation conditions for co-culture of the bacterium together with the host fungi are also very important factors. The concentration of acetosyringone, a wound response molecule required for activation of the *vir* gene [83], is normally maintained at 0.2 mM for most of the mushroom transformation (Table 3). Duration and temperature for co-culture are normally 2~5 days and near 25°C, respectively, but can last up to a month at low temperature for slow-growing mushrooms [74, 75, 78, 80]. Various binary vectors containing T-DNA regions, which are composed of

Table 3. Selected mushroom transformation by ATMT

Host		<i>Agrobacterium</i> strain	Co-culture conditions	Vector				Reference
Species	Cell type			Plamid name	Promoter	Reporter gene	Marker (µg/mL ^a)	
<i>Agaricus bisporus</i>	Protoplast	LBA1100	25°C, 5 days	pTAS10	<i>A. niger</i> gpd		hph (25)	[61]
	Tissue	AGL-1	25°C, 3 days	pBGgHg	<i>A. bisporus</i> gpd	eGFP	hph (50)	[64]
<i>Agaricus bisporus</i>	Protoplast	LBA1100	25°C, 5 days	pUR5750	<i>A. niger</i> gpd		hph (30)	[65]
	Tissue	AGL-1	25°C, 2~3 days	pGRhph004	<i>A. bisporus</i> gpd	GFP	hph (50)	[66]
	Tissue	LBA1126						
	Tissue	AGL-1	25°C	pBlue	<i>A. bisporus</i> spr1	GFP	hph (25)	[67]
	Tissue	AGL-1	25°C, 3 days	pBHg	<i>A. bisporus</i> gpd	Pabs	hph (50)	[68]
<i>Coprinopsis cinereus</i>	Protoplast	AGL-1		pUGiGM3	<i>C. cinerea</i> trp1		Trp	[66]
	Protoplast	LBA1126						
<i>Cordyceps militaris</i>	Conidia	AGL-1	23°C, 2 days	pATMT1	<i>A. niger</i> trpC		hph (400)	[69]
<i>Flammulina velutipes</i>	Tissue	AGL-1	25~28°C, 3 days	pBGgHg	<i>A. bisporus</i> gpd	eGFP	hph (50)	[70]
	Tissue	AGL-1	25~28°C, 4 days	pBGgHg	<i>A. bisporus</i> gpd	eGFP	hph (30)	[71]
	Mycelium	AGL-1	22°C, 4 days	pFHC	<i>A. niger</i> trpC <i>F. velutipes</i> gpd	Cob	hph (10)	[72]
<i>Ganoderma lucidum</i>	Protoplast	LBA4404	25°C, 3.5 days	pGL-GPP	<i>G. lucidum</i> gpd	GUS	hph (100)	[73]
<i>Hypsizygus marmoreus</i>	Mycelium	EHA105	2~4°C, 28 days	pPZP-HyG2	<i>C. neoformans</i> actin		hph (100)	[74]
	Protoplast	EHA105	1~2°C, 26 days	pHm-GPD	<i>H. marmoreus</i> gpd	eGFP	hph (20)	[75]
<i>Laccaria laccata</i>	Mycelium	LBA4404						
<i>Laccaria laccata</i>	Mycelium	AGL-1	22°C, 5 days	pBGgHg	<i>A. bisporus</i> gpd	eGFP	hph (120)	[76]
<i>Pleurotus eryngii</i>	Tissue	GV3101	25°C, 7~14 days	pCAMBIA1304	CaMV 35S	IL-32	hph (50)	[77]
<i>Pleurotus eryngii</i>	Protoplast	AGL-1	1°C, 25 days	pBGgHg	<i>A. bisporus</i> gpd	eGFP	hph (30)	[78]
<i>Pleurotus ostreatus</i>	Spore	AGL-1	25°C, 3 days	pPEH	<i>P. ostreatus</i> gpd		hph (50)	[79]
	Mycelium	GV3101						
<i>Suillus bovinus</i>	Tissue							
<i>Suillus bovinus</i>	Mycelium	LBA1100	4°C, 20 days	pBIN19-17	<i>S. commune</i> gpd		hph (200)	[80]
<i>Volvariella volvacea</i>	Spore	EHA105	3°C, 28 days	pLg-afp235	<i>L. edodes</i> gpd	Afp		[81]
	Mycelium	LB4404						
<i>Volvariella volvacea</i>	Tissue							

^aConcentration of hygromycin.

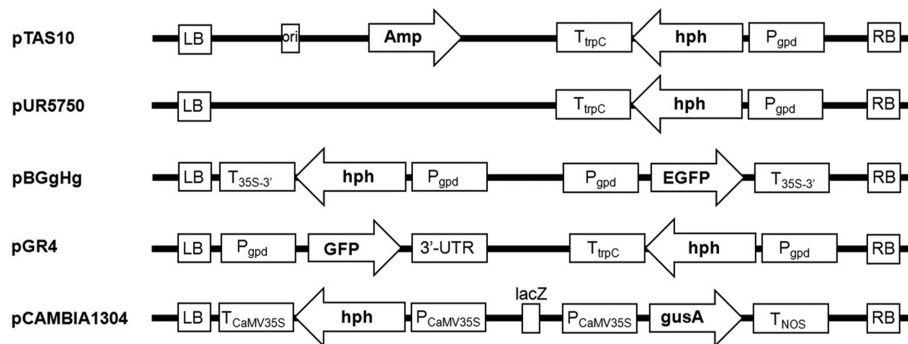


Fig. 1. T-DNA regions of selected binary vectors. The binary vectors and related citations are described in Table 3. LB and RB are the left board and right board of T-DNA region in Ti plasmid, respectively.

insert DNAs flanked by a left board and right board, have been applied to mushroom transformation. The T-DNA regions mostly convey bicistronic genes, one for the selective marker and another for the reporter gene or gene of interest, whose expression is regulated by independent promoters (Fig. 1). The most frequent selective marker is a hygromycin resistant gene (*hph*), due to lack of auxotrophic mutant strains. *C. cinereus* is one of the few mushrooms which can use an auxotrophic marker [66, 67]. Expression of the reporter or selective marker gene is regulated by constitutive promoters, such as glyceraldehyde-3-phosphate dehydrogenase promoter (P_{gpd}) and cauliflower mosaic virus 35S promoter (P_{CaMV35S} or P_{35S}), and terminators, such as T_{trpC} and T_{35S} . More rigorous efforts should be made to generate mutant strains with robust selective markers and to identify controllable promoters.

Integration of linear DNA by REMI. REMI is a transformation method for introducing linear DNA, which is cut by certain restriction enzyme(s), into the same restriction site in the host genome by including linear DNA together with the restriction enzyme in the transformation mixture [84]. REMI is a powerful tool to transform a variety of filamentous fungi [81, 85] and mushrooms, including *C. congregatus* [30], *F. velutipes* [31], *P. ostreatus* [40, 42], *P. eryngii* [37], *Trametes versicolor* [45], *Ganoderma lucidum* [32], and *L. edodes* [33-36]. For example, we previously transformed a dikaryotic strain of *P. eryngii* with *Hind*III-digested pAN7-1-EGFP fragment using PEG4000-treated protoplasts in the presence of *Hind*III [37] (Fig. 2). Although the transformation was successful as shown by the expression of EGFP in the mycelia, we failed to detect EGFP protein in the mature fruiting bodies. The integrant

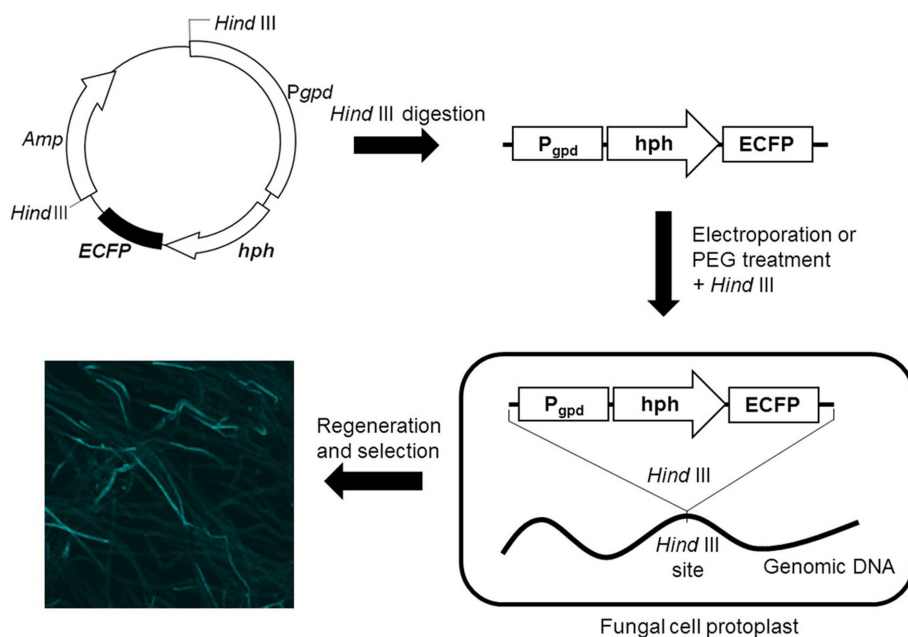


Fig. 2. Schematic description for restriction enzyme-mediated integration (REMI). The figure shows application of REMI to introduce *hph*-EGFP gene into the genomic DNA of *Pleurotus eryngii* [37]. EGFP expression was observed under fluorescence microscope.

was eventually diluted out during subculture. This may indicate that direct integration of foreign DNA into one of the nuclei in dikaryotic cells generates a fairly unstable transformant, suggesting that it is more desirable to use a monokaryotic strain in any mushroom transformation. Until now, REMI has been conducted on protoplasts of mushroom cells treated with PEG3350 or PEG4000 [30-37, 40, 42]. Restriction enzymes employed for REMI are mostly six-cutter enzymes, such as *EcoRI* [30], *HindIII* [37], and *BamHI* [42].

CONCLUSIONS

Introduction of a foreign gene into living cells and deletion of a target gene from genomic DNA are essential steps for better understanding cell biology at the molecular level. Nevertheless, mushroom transformation has not been freely accessible partly due to the dikaryotic nature of mushroom mycelia. More importantly, mushroom cells have their own limitations as good hosts for foreign DNA since HR is suppressed in filamentous fungi, whereas NHEJ is the major repair system against DSBs. With NHEJ, integration of foreign DNA has to be random, which makes it difficult to target a specific gene. Therefore, suppression of NHEJ by downregulation or knockout of NHEJ components is necessary and conceivably a prerequisite. Filamentous growth with connected cells is another obstacle since it prevents isolation of single cells from certain experimental treatments and thus causes difficulties in the selection of independent cells with a desirable phenotype. Use of germinating spores, protoplasts, and finely broken mycelia with regenerated apical tips may circumvent this problem. Generation of a variety of auxotrophic mutant strains is also needed to make mushrooms as an efficient host as yeast. In closing this review, more serious efforts should be concentrated on the host engineering for further advances in mushroom science.

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