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Genetic transformation of fungi

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ABSTRACT Transferring DNA into cells is an essential research method for molecular cloning and gene function studies. As molecular biology and materials physics develop, more and more new transformation methods have been applied to mammalian cells. Some techniques have been successfully developed for several types of fungi, but their efficiencies are extremely low. To better study the functional genes of fungi, and to improve the characteristics of fungi in an easy, safe and reliable way, many investigations have been conducted to effectively develop such technologies for a wide variety of species and to increase the efficiency and reproducibility of genetic transformation. The objective of this paper is to review the latest development of transformation methods used for the genetic transformation of fungi, including several promising transformation approaches, together with their advantages and drawbacks, which may open up novel methods for fungi research.

KEY WORDS: *genetic transformation, fungi, electroporation, liposome, nanomaterial*

Introduction

Fungi, widespread eukaryotes in nature, connects with the natural environment, humans and other creatures extensively via saprophytism and symbiosis, including yeasts (unicellular microorganisms) and filamentous fungi (multicellular microorganisms), as well as multicellular fungi that produce familiar fruiting forms known as mushrooms (Fig. 1). Some species play an important role in such diverse fields as medicine, agriculture and industry. Apart from the species of biotechnical or pharmaceutical significance, many fungi are also pathogenic in plants, animals and humans. Especially, as the organ transplantation develops and the abuse of immunosuppressive agents and corticosteroids increase, the fungal infection rates have also increased sharply, which becomes one of the main causes of death of patients suffering from severe immune damages. One major area of current research regarding fungi focuses on delineating their molecular mechanisms of pathogenesis. The development of an effective DNA transformation approach for fungi is very critical in order to identify the gene function and improve its genetic characters.

Since the first report of transformation in *Neurospora crassa* in 1973, a lot of methods have been developed to transfer DNA into about 100 species in all major groups of fungi, including filamentous fungi and yeast. However, these methods have some drawbacks, such as intricate procedures, high-priced instruments and extremely low transformation rates, which motivates search for more efficient, simpler and safer techniques facilitating the uptake of exogenous DNA into host cells. The cell wall of fungi is the main hindrance for a successful transformation. Popular and promising tools used for genetic transformation of fungi, including *Agrobacterium tumefaciens*-mediated transformation (ATMT), electroporation, viral vectors-mediated transformation, liposome-mediated transformation and nanomaterial-mediated transformation, are described in this paper.

Traditional methods

It has been shown that protoplasts made from young hyphae, mycelium and conidiospore (Fig. 1) are the ideal recipient materials for fungal genetic transformation. Since the first report of transformation in *Neurospora crassa* by using PEG/CaCl2-mediated protoplast in 1979(Case *et al*., 1979), this method has been successfully applied in many other fungi, such as *Aspergillus fumigates* (Yu *et al*., 2015), *Penicillium purpurogenum* (Kojima *et al*., 2015), and *Acremonium implicatum* (Yao *et al*., 2015). However, the complicated procedures and the low regeneration rate limit its application, especially for some species, due to the low yield of protoplasts that can be produced (Fig. 2A). A lithium acetate-mediated transformation does not depend on a protoplast. A certain concentration of Li^+ will increase the permeability of cells and allow exogenous DNA to penetrate cell walls, increasing transformation efficiency. Recently,

Abbreviations used in this paper: ATMT, *Agrobacterium tumefaciens*-mediated transformation; GFP, green fluorescent protein; REMI, restriction enzyme-mediated integration; TMV, tobacco mosaic virus.

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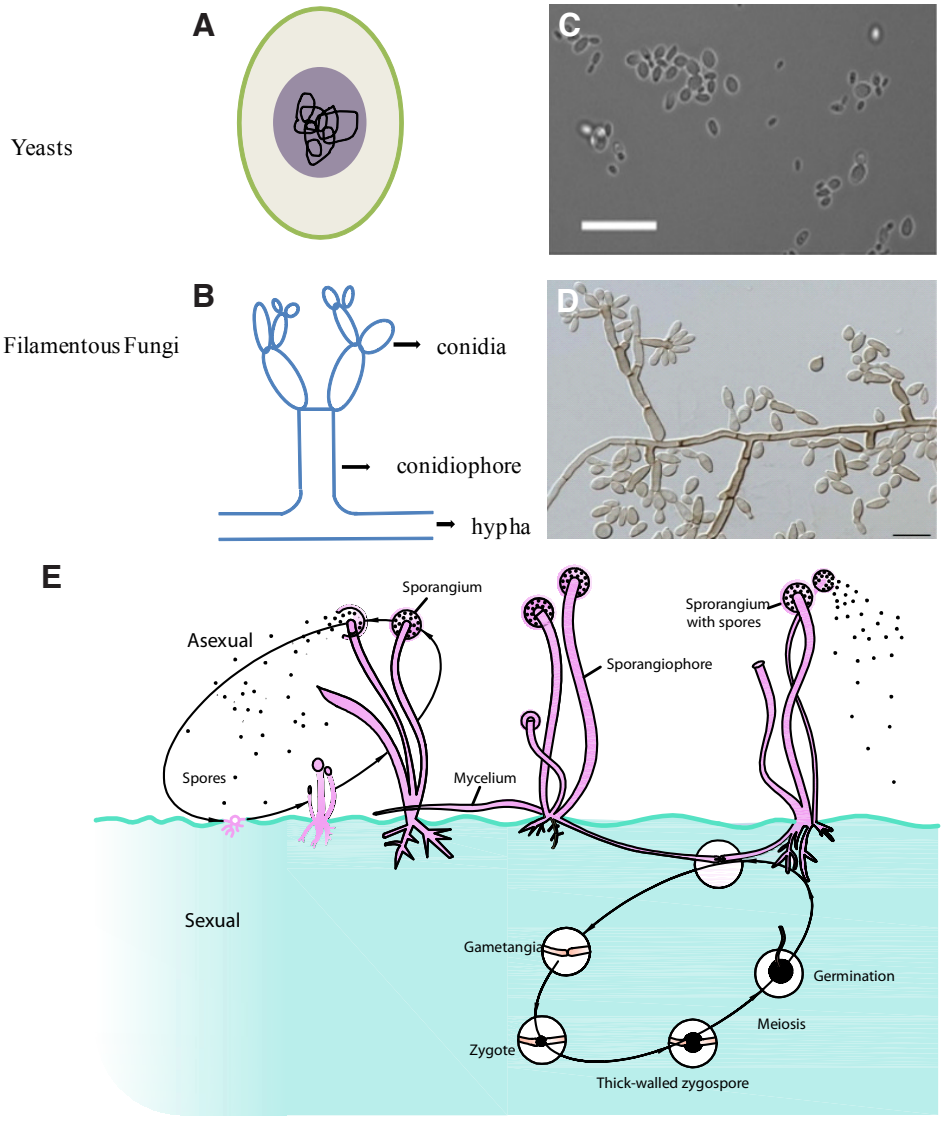

Fig. 1. Life cycle of fungi. (A,B) *The structure of the yeast and filamentous fungi; yeast are unicellular microorganisms* **(A)**, *whereas filamentous fungi are multicellular microorganisms (generally including conidia, conidiophore and hypha)* **(B). (C,D)** *The microscopic morphology of* Candida pseudoaaseri **(C)** *(yeasts) (Pfuller* et al.*, 2011) and* Fonsecaea pugnacious **(D)** *(filamentous fungi) (de Azevedo* et al.*, 2015).* **(E)** *The sexual and asexual reproduction of fungi.*

Calvey *et al.,* (2014) reported the development of an efficient genetic transformation system for *Lipomyces starkeyi* based on a modified lithium acetate transformation protocol, and achieved efficiencies in excess of 8,000 transformants/μg DNA (Calvey *et al.*, 2014). Other modifications of the lithium method include the addition of polyethylene glycol (PEG) (Cartwright *et al.*, 2014), dithiothreitol (DTT), and dimethyl sulfoxide (DMSO). Nevertheless, limited numbers of species in fungi have been transformed by lithium acetate mediated transformation (Rivera *et al.*, 2014). Restriction enzyme-mediated integration (REMI) transformation, based on the PEG-mediated transformation of protoplast, is a common method to transfer nonhomologous linearized DNA into chromosomes of host cells mediated by numerous fungal pathogens. Chen *et al.,* (2014) cloned the cytochromeP450 reductase (*CPR*) gene from into *Rhizopus nigericans* to strengthen the expression of *CPR* gene in *R. nigericans* with REMI, and the highest biotransformation rate of the transformation was 65.38%. Regardless, the high rate of mutations independent of the transferred DNA and the dependence on transformation utilizing protoplasts limits these techniques for application in fungi (Chen *et al.*, 2014). Biolistics is the physical method which is relatively more efficient technique used to increase the accessibility of targeting DNA fragments into host cells (Parveez *et al.*, 2015). The improvement of targeted delivery using biolistics has shown to be successful in fungal transformation(Djulic *et al.*, 2011; Kushawaha *et al.*, 2015), as the DNA is directly shot into the living cells using super-speed gold or tungsten particles for host cell uptake. Mutants obtained through biolistics transformation exhibited increased genetic stability (Teixeira Da Silva *et al.*, 2016).

Nevertheless, expensive equipment and tedious optimization of various factors influencing transformation efficiency make it an uncommon method of choice in most laboratories. Furthermore, electroporation and *Agrobacterium tumefaciens*-mediated transformation (ATMT) are also the classical and high-efficiency systems for genetic manipulation in fungi, which will be discussed later in detail.

Electroporation

Electroporation is the most common physical technique for fungal transformation, which was first applied in 1989 (Delorme, 1989). From then on, this approach is becoming more and more popular, because it is unsophisticated, expeditious and efficient, even though it requires laborious protocols for regeneration after genetic transformation (Biswas, 2015). It is based on the application of strong electrical fields to cells or tissues and utilizes the pulse electric field transient shock recoverability of receptor cells, which leads to the formation of a cell membrane reversible instantaneous pore channel. These pores are large enough to allow the passage of DNA, protein and DNA–protein complexes from the outside milieu into the cells (Fig. 2B). Studies have shown that the efficiency of electroporation is higher than protoplast (Gu *et al.*, 2015). The transformants of electroporation are always genome randomly assigned, which is more suitable for the transformation of filamentous fungi (Vela-Corcía *et al.*, 2015). Additionally, appropriate adjustment of the physical parameters and special fungal cell treatments are required to establish a specific protocol for each species (Simonis *et al.,* 2017).

Modifications of the electroporation method including the addition of polyethylene glycol (PEG), dithiothreitol (DTT), and dimethyl

sulfoxide (DMSO) can effectively improve efficiency. Miklenić *et al.,* (2015) reported an optimization of electroporation procedure which resulted in significant increase of transformation efficiency (from 10-20 transformants μg^{-1} to $2.8x10^3$ transformants μg^{-1}). Several key transformation parameters were optimized including cell growth phase, density of cells in the transformation sample and electroporation settings. And they found that treating the cells with both lithium acetate (100 mM) and dithiothreitol (35 mM) synergistically improves transformation efficiency (Miklenić *et al*., 2015).

There are several shortcomings for electroporation. The application of an electric field in combination with chemical treatments raises the rate of cellular death. The transformation of intact cells has only been reported in a few species. Furthermore, manipulation of physical parameters such as the electric field strength is not correlated with high frequencies of transformation (Hao *et al*., 2015). These disadvantages make electroporation less useful for transforming filamentous fungi.

Agrobacterium tumefaciens-mediated transformation (ATMT)

Agrobacterium tumefaciens, a gram negative plant pathogenic bacterium, can cause crown gall in plants. The tumor-inducing (Ti) plasmid DNA in *A. tumefaciens* is capable of transferring a piece of its DNA into host cells, where it is integrated into the host chromosome and expressed (Fig. 2C). For these reasons, *Agrobacterium tumefaciens* is a natural vector for genetic delivery.

Agrobacterium tumefaciens-mediated transformation (ATMT) used in fungi was first reported in 1990s (de Groot *et al*., 1998). Until now, a wide variety of different fungal species have been transformed using this method, with *Ustilaginoidea virens* (Yu *et al.,* 2015), *Lecanicillium lecanii* (Zhang *et al.,* 2014), and *Paracoccidioides brasiliensis* (Marcos *et al.,* 2016) as some of the last examples. Researchers have optimized a range of conditions to improve the transformation efficiency (Hu *et al*., 2014).

ATMT is a popular approach for the genetic transformation of fungi because it has several advantages. Firstly, different types of tissue, such as conidia, mycelium or even fruiting bodies, can be transformed by ATMT (Mora-Lugo *et al.,* 2014; Zheng *et al.,* 2017). Thereby, it avoids the enzymatic treatment and tedious process of protoplast preparation. Secondly, the T-DNA can be randomly inserted into the host genome, typically as a single copy, and showing a greater degree of stability of the transgene (Zhang *et al*., 2014; Gong *et al*., 2015). Last but not least, compared with other transformation techniques, the ATMT method has been proven to increase transformation rate. Therefore, the ATMT system offers

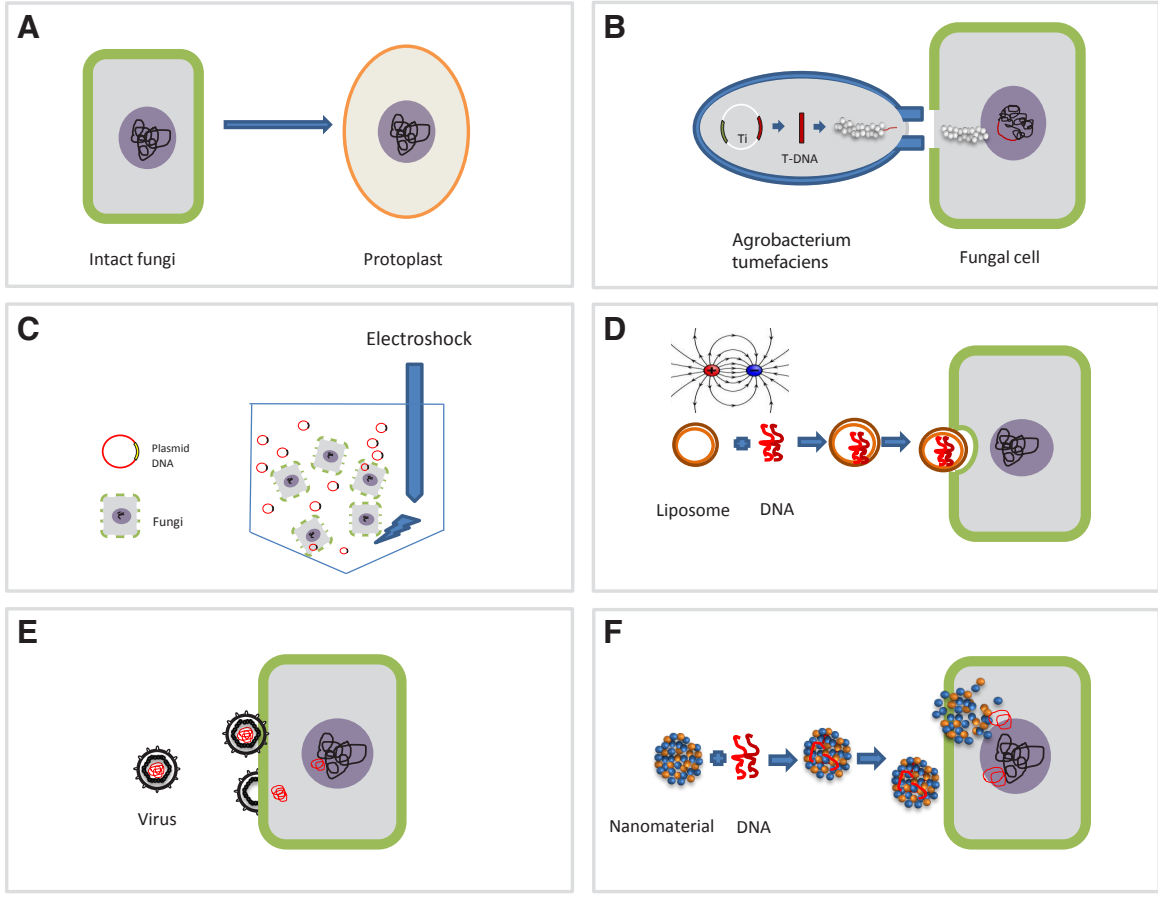

Fig. 2. Transformation methods in fungi. *Traditional methods always require protoplast formation* **(A)**, *which is very complicated and requires different enzymes to dissolve the cell walls of fungi. Electroporation, utilizing strong electrical fields to cells or tissues, which leads to the formation of a cell membrane reversible instantaneous pore channel. These pores are large enough to allow the passage of DNA, protein and DNA–protein complexes from the outside milieu into the cells* **(B)**. Agrobacterium tumefaciens **(C)**, *liposome* **(D)**, *viral vectors* **(E)** *and nanomaterial* **(F)** *can also be used to carry DNA into fungi.*

an efficient tool for random molecular manipulation.

However, ATMT is time-consuming and prone to low transformation rate due to various factors, including acetosyringone concentration (Tzima *et al.*, 2014), co-culturing time, co-culturing temperature and fungal inoculums, which were found to significantly impact the transformation frequency (Sorensen *et al.*, 2014). The research about how the T-DNA is transferred into the yeast cells is on the way. Luo *et al.,* (2015) developed genetic screening of yeast knockout mutants and identified a yeast actin-related protein ARP6 as a negative regulator of ATMT (Luo *et al.*, 2015). Their experiments showed that ARP6 is a negative regulator of the ATMT process as knockout of ARP6 consistently and significantly increased transformation efficiency.

Liposome-mediated transformation

Composed of a phospholipid bilayer, liposomes are tiny, synthetic lipid vesicles that can be used as a tool to facilitate the delivery of exogenous DNA during a transformation (Fig. 2D) (Yu *et al.*, 2015). Liposomes contain hydrophilic head groups, hydrophobic tails, and attain positive charges. They form bimolecular lipid vesicles in an aqueous environment. Negatively charged DNAs can be attracted and engulfed in the vesicles, which protects the DNAs from degradation by nucleases (Chen *et al.*, 2015). Cationic liposomes spontaneously associate with negatively charged DNA, and thereby form lipoplexes, which is the first step in the transfection process. DNAs enter cells via endocytosis of the liposomes which adhere and fuse with the negatively charged cell membrane (Radaic *et al.*, 2015).

Liposomes based on cationic lipids have been favored for many potential advantages compared with other non-viral vectors, for their excellent biocompatibility, low immunogenicity, large nucleic acid packaging capacity and large-scale production (Govender *et al.*, 2015). For these reasons, it is broadly used for gene delivery in gene therapy (Wang *et al.*, 2015).

Liposome-mediated transformation is widely applied in cells with no cell wall. However, the usage in cells with walls, such as bacteria, fungi, and plants, was very limited. The successful application of this method in fungi has shown that liposome-mediated transformation could be an effective, fast, and simple transformation method for fungi (Chai *et al.*, 2013). CHAI *et al.,* (2013) developed a procedure to introduce DNA into mycelium of filamentous fungi, *Rhizopus nigricans* LH21 and *Pleurotus ostreatus* TD 300, by liposome-mediation but with no protoplast preparation. Their study has shown that the transformation efficiencies were similar to those of electroporation-mediated protoplast transformation (EMPT) of *R. nigricans* or PEG/CaCl2-mediated protoplast transformation (PMT) of *P. ostreatus*, respectively.

Nonetheless, liposome-mediated transformation also has some defects, including high toxicity (Chen *et al.*, 2015), less transfection efficiency, and *in vivo* instability (Khatri *et al.*, 2014). Some modifications overcome these drawbacks. Ju *et al.,* (2015) discovered a novel cholesterol-based cationic lipid, which represented a potential agent for the liposome used in gene delivery due to low cytotoxicity and impressive gene transfection activity (Ju *et al.*, 2015).

The two types of factors that influence the successful introduction of exogenous molecules by liposome-mediated transformation are: lipid composition and cell type. Liposome formulations with different 1, 2-dioleoyl-3-trimethylammonium propane (DOTAP) to dioleoylphosphatidylethanolamine (DOPE) weight ratios (Kim *et al.*, 2015) show different efficiency. But the factors of size or zeta potential have not been proven to affect the efficiency.

Viral vector-mediated transformation

Gene delivery vectors are classified into viral and non-viral ones (Salazar-Montes *et al.*, 2015). Among them, viral vectors are an efficient means for gene delivery. They are able to mediate efficient gene transfer with long-term gene expression.

Plant and fungal viruses can infect fungi. Plant viruses have proven to be quite versatile as tools for biotechnology. They have been used as vectors for protein expression in plants and fungi, as well as for expression of fragments of plant genes to silence endogens (Fig. 2E) (Mascia *et al.*, 2014). The ability to use fungal viruses for gene delivery in fungi is more limited, due to a number of factors, including the less developed tools available, the less amenable systems for transfection of many fungi, and stability of the fungal virus vectors (Li *et al.*, 2015). Fortunately, it is good news that plant viruses can infect fungi.

Tiziana Masci *et al*(Mascia *et al.*, 2014) provided evidence that a recombinant tobacco mosaic virus (TMV) vector carrying a gene for the ectopic expression of the green fluorescent protein (GFP) is able to induce the stable silencing of the GFP in the *Colletotrichum acutatum* transformant line 10 expressing GFP derived from *C. acutatum* (strain C71). The TMV-based vector also enabled *C. acutatum* to transiently express exogenous GFP up to six subcultures, without the need to develop transformation technology. Isolated from a plant pathogenic ascomycete, white root rot fungus, Rosellinia necatrix victorivirus 1 (*RnVV1*) (Shimizu *et al.*, 2015; Yaegashi *et al.*, 2016) was regarded as a novel victorivirus. The virus was molecularly and biologically characterized using the natural and experimental hosts (chestnut blight fungus, *Cryphonectria parasitica*) and was shown to have typical molecular victorivirus attributes. Zhai *et al.,* (2015) isolated and sequenced another victorivirus, called "Botryosphaeria dothidea victorivirus 1" (BdV1), from *Botryosphaeria dothidea.* The predicted gene product of ORF1 had the highest amino acid sequence similarity to the capsid protein of Rosellinia necatrix victorivirus 1 (RnV1), and ORF2 had the highest similarity to Beauveria bassiana victorivirus (Zhai *et al.*, 2015). It is theoretically feasible that victorivirus could realize transformation without the need to develop transformation technology.

Although viral vectors have many advantages, some side effects, including safety concerns of an acute immune response, immunogenicity, and insertional mutagenesis limit its application (Ramamoorth *et al.*, 2015). Finding nontoxic virus carriers is the key to the breakthrough for the development of this technology to be widely used.

Nanomaterial-mediated transformation

Nanomaterial-mediated gene delivery systems are gaining recognition as alternative to viral gene vectors due to their safety and low immunogenicity (Chira *et al.*, 2015). The effective delivery of nucleic acids requires overcoming many biological barriers that would otherwise hinder transfection efficiency (Priyadarshani *et al.*, 2016). Developing safe and efficient vectors that can overcome

TABLE 1

COMPARISON OF METHODS FOR FUNGAL GENETIC TRANSFORMATION

Methods	Advantages	Disadvantages
PEG/CaCl2-mediated protoplast	Different cell types (conidia, mycelium) can be used.	Complicated procedures and low generation rate of protoplasts.
Lithium acetate-mediated transformation	Does not depend on a protoplast.	Complicated procedures.
REMI transformation	Effective technique.	High rate of mutations independent of the transferred DNA and the dependence on transformation utilizing protoplasts.
Biolistics	No pretreatment of the cell wall required. Independent of the physiological properties of the fungi.	Expensive equipment and tedious optimization of various factors.
Electroporation	Simple, fast and cheap method. Genome randomly assigned.	Raises the rate of cellular death. Depends on the electrophysiological characteristics of the fungus.
Agrobacterium tumefaciens-mediated transformation (ATMT)	Different cell types (conidia, mycelium) can be used. Not requires protoplast formation.	Time-consuming. Requires tedious optimization of various factors during co-cultivation.
Liposome-mediated transformation	Effective, fast, and simple transformation method.	High toxicity, less transfection efficiency, and extracellular instability of the delivery complex.
Viral vectors-mediated transformation	Effective, fast, and simple transformation method.	Highly immunogenic, relatively difficult to product.
Nanomaterial-mediated transformation	Fast and simple transformation method, safety and low immunogenicity.	Limited used, less transfection efficiency.

these obstacles is at the heart of current gene delivery research.

Nanomaterial-mediated gene delivery technologies have been developed using layer-by-layer self-assembly of nanomaterials held together by electrostatic interactions in order to provide nanoparticulate materials that protect DNA and deliver DNA into cells (Fig. 2F)(Nafissi *et al*., 2015). Polymeric nanocarriers, such as polyethylenimine (PEI), poly (L-lysine) (PLL), poly [2-(dimethylamino) ethyl methacrylate] (PDMAEMA), polyamidoamine (PAMAM), chitosan and poly (amino-co-ester) s (PAEs), play an important role in gene delivery. Polymeric nanocarriers always combine with other materials, which include magnetic nanoparticle (Pivetal *et al*., 2014), grapheme oxide (Varela *et al*., 2014), and silver (Mishra *et al*., 2015), to make gene delivery more efficient.

Fungal cells can be genetically transformed by non-viral polymeric nanocarriers in the presence of carrier and plasmid DNA (Tanasienko *et al*., 2015). Using a novel nanoscale oligoelectrolyte polymer possessing a comb-like structure as a carrier molecule, Filyak *et al.,* (2013) transformed exogenous DNA into *Saccharomyces cerevisiae* via DMAEM-based cationic polymers (Filyak *et al*., 2013).

After making comparisons with standard transformation methods, a yield of two times more transformants of *Hansenula polymorpha NCYC 495* compared to electroporation approaches, their results clearly indicate that genetic transformation of yeasts using oligoelectrolyte polymer carriers is a highly effective means of gene delivery. Filyak *et al.,* (2015) used a new nanoscale comb-like oligoelectrolyte polymer for plasmid DNA delivery into the yeast cells. The nanoscale comb-like oligoelectrolyte polymer combines an anionic backbone and dimethyl aminoethyl methacrylate (DMAEM)-based side branches for DNA delivery into yeast cells of *Pichia pastoris, Hansenula polymorpha* and *Saccharomyces cerevisiae*. Their results show that the new method is more efficient than the lithium acetate method and electroporation (Filyak *et al*., 2015).

Although significant progress has been made in the development of non-viral gene delivery system, the majority of the non-viral approaches remain significantly less efficient than viral vectors (Li *et al*., 2015).

Conclusions and perspectives

Fungal genetic transformation is essential for study on functional genes. Transformation of exogenous sequences into host cells will produce random mutants, which can help researchers improve the characteristics of fungi in an easy, safe and reliable way. Over the past decades, substantial achievements have been made in different areas related to genetic transformation, including development of new technologies and novel delivery materials, as well as improved efficiency and stability of transformants.

However, at present, the genetic transformation of fungi, whether performed by classical or other novel methods, still faces major challenges. Better understanding of the phenomena involved in genetic transformation can help make protocols more convenient and rigorous. Additionally, methods of genetic transformation of fungi are not separated. In order to improve the efficiency of transformation, an overall consideration of the advantages and drawbacks of different methods can be taken and they can be combined with each other.

As the researches on molecular biotechnology of fungal are gradually improved, more and more fungal molecular mechanisms will be understood. Our review for the application of these methods in fungi hopes that subsequent scientific researches and in-depth studies will focus on these new methods. Therefore, the progress of genetic transformation will create new opportunities for forward and reverse genetics in fungi and will promote the fungus to have more favorable and far-reaching impact on human's life activities.

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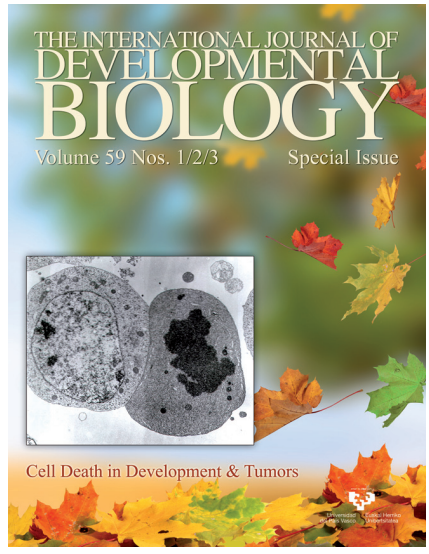

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