Fungal Biology

Marco A. van den Berg
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Genetic Transformation Systems in Fungi, Volume 1

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Preface

Fungi are a highly versatile class of microorganisms and their habitats are as diverse. In nature, fungi play a crucial role in a range of degradation processes, enabling recycling of valuable raw materials by wood decaying fungi like the white rot fungus *Phanerochaete chrysosporium*. On the other hand, fungi can be pests to food production like the rice blast fungus *Magnaporthe oryzae*. Furthermore, mankind exploits the enzymatic opportunities of fungi through classical industrial processes as ethanol production by the yeast *Saccharomyces cerevisiae* and heterologous enzyme production by filamentous fungi as *Trichoderma reesei*. All these stimulated an enormous number of studies trying to understand as well as exploit the metabolic capabilities of various fungal species.

One of the game-changing breakthroughs in fungal research was the development of genetic transformation technology. This enabled researchers to efficiently modify the gene content of fungi and study the functional relevance. Interestingly, the first available method (protoplast or spheroplast transformation) evolved from an existing classical method called protoplast fusion, a process which also introduces DNA into a receiving cell however in an uncontrolled way.

This publication aims to give an overview of all existing transformation methods used for yeasts and fungi. It is meant not only as reference material for the experienced researcher but also as introduction for the emerging scientist. Therefore, all methods are supported by several illustrative example protocols from various fungal species and laboratories around the world, which will be a good starting position to develop a working protocol for other fungal species being studied.

Transformation methods do not describe the whole story; DNA must enter the cell, the nucleus and finally integrate the genome, if required also at predetermined positions. By including associated methods and tools as cell fusion, repetitive elements, automation, analysis, markers, and vectors this volume reflects the many relevant elements at hand for the modern fungal researcher.

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Part I

Introduction

Fungal Transformation: From Protoplasts to Targeted Recombination Systems

1

Juan F. Martín

1.1 Introduction

Yeast and filamentous fungi show an impressive metabolic diversity and play very important roles in nature and in the activities of the human society. Briefly, fungi are involved in many diverse degradative processes in nature; yeast are particularly notorious for their ability to ferment some sugars to ethanol, and several other filamentous fungi are excellent producers of a variety of hydrolytic enzymes. Several basidiomycetes (fruiting bodies) are edible and other fungi are involved in food ripening processes (Machida et al. 2005; Fernández-Bodega et al. 2009). Finally, filamentous fungi produce an impressive array of secondary metabolites with useful pharmacological activities. Some of the secondary metabolites are extremely toxic to humans and animals (mycotoxins).

1.1.1 A Historical Perspective of Transformation of Fungi

It is now about 30 years since the first articles on transformation of a few filamentous fungi were published (Buxton and Radford 1983; Tilburn et al. 1983; Yelton et al. 1984; Ballance et al. 1983; Ballance and Turner 1985; Saunders et al. 1986; Cantoral et al. 1987). Previously, transformation of the yeast *Saccharomyces cerevisiae* had been well established (Hinnen et al. 1978; Williamson 1985) but attempts to use some of the yeast replicating plasmids (e.g., YRp10) and yeast genes to complement filamentous ascomycetes were unsuccessful (Cantoral et al. 1987) and therefore it was necessary to develop entirely new vectors and transformation procedures.

Hundreds of articles using transformation of fungi, as a tool for fungal genetics, have been published since then but it is surprising how little general information on transformation procedures, common to all fungi, has been established on solid grounds. Transformation of different fungi is generally a tricky process and frequently each research group has developed its own protocols. In this introductory chapter I will not make a comprehensive review of all described transformation procedures. Rather I will summarize the initial trials until a reliable transformation procedure was set for a few model fungi, including *Neurospora crassa* (Yelton et al. 1984), *Aspergillus nidulans* (Ballance et al. 1983; Ballance and Turner 1985), *Acremonium chrysogenum* (Skatrud et al. 1987), or *Penicillium chrysogenum* (Cantoral et al. 1987) among others. Detailed analysis of the transformation procedures of many other fungi are given in other chapters of this book. Relevant findings that contributed significantly to the development of fungal transformation systems are reviewed here in more detail.

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There are hundreds of fungal species, including the ascomycetes, basidiomycetes, and zygomycetes but transformation of only some of them has been achieved. Each species may require optimization of protocols or, even in some cases, development of entirely novel procedures.

Interestingly, different natural isolates or highly mutated industrial strains (so called "domesticated strains") (Machida et al. 2005; Jami et al. 2010a, b), have acquired changes in the cell wall and it is frequently very difficult to obtain protoplasts of those strains and transformants using the protocols and conditions optimized for the wild type strains.

Although transformation of many ascomycetes has been achieved, a more difficult situation is found in many basidiomycetes or phycomycetes. In some cases the extreme difficulty to obtain transformants of these fungi has been solved by the use of *Agrobacterium tumefaciens*-mediated conjugation, e.g., transformation of *Agaricus bisporus* or *Hypholoma sublateritium* (de Groot et al. 1998; Godio et al. 2004).

1.2 Early Development of Basic Tools for Transformation

1.2.1 Protoplasts and Lytic Enzymes

The transformation of filamentous fungi relies largely on the efficient preparation of fungal protoplasts, although some alternative transformation methods exist that do not require protoplasts (see below). However, the reliable preparation of fungal protoplasts has proven to be difficult, because of the poor knowledge on lytic enzymes that digest the cell wall of fungi.

Fungal cell walls are composed of glucans, mannans, and chitin that form a sac-like tridimensional structure of microfibers linked to cell envelop proteins. The proportion of the different cell wall polymers is distinct for each species of fungi, and differs between spores and hyphae cell walls (Martín et al. 1973). This different composition hampers the standardization of the conditions for optimal preparation of protoplasts.

Authentic protoplasts are defined by three characteristics described by Villanueva and García-Acha (1971), namely (1) protoplasts are spherical cells, lacking cell walls, surrounded by the plasma membrane, (2) they are released leaving empty "ghost" cell walls, and (3) they are viable but osmotically sensitive and therefore, require an osmotic stabilizer. Cell wall-free true protoplasts are sometimes difficult to obtain and the term sphaeroplasts was used by these scientists to refer to spherical cells that still carry remnants of cell wall polymer fragments. They are also osmotically sensitive and the presence of cell wall remnants may be favorable to start cell wall regeneration (acting as primers of cell wall polymers initiation).

Formation of *S. cerevisiae* protoplasts by *Helix pomatia* intestinal juice was reported in the 1950s. This juice contains a cocktail of carbohydrate-hydrolytic enzymes including glucanases, glucuronidases, and arylsulphatases, among others. A commercial preparation of the *H. pomatia* juice with the name of Glusulase was used by several research groups to obtain yeast protoplasts, although the *H. pomatia* juice seems to lack other enzymatic activities required for efficient release of protoplasts from filamentous fungi. Studies on lytic enzymes of bacterial and fungal origin at the Universities of Salamanca and Nottingham (Gascón and Villanueva 1964; Peberdy 1979) led to the use of lytic enzyme mixtures from the culture broth of a few actinobacteria (e.g., *Streptomyces graminofaciens, Micromonospora chalcea*) and fungi (particularly *Trichoderma harzianum*). A major problem of these lytic preparations was the limited reproducibility of the activities in the culture broths. Since then, commercial preparations of the lytic enzymes were made available (e.g., Novozym 234) and were widely used.

The addition of glucanases of different origins and laminarinase of actinomycetes grown on laminarin obtained from brown algae has also been tested (Gascón and Villanueva 1964). In many cases the excess of lytic enzymes or the presence of poorly characterized phospholipases is clearly damaging for the protoplasts stability even in the presence of osmotic stabilizers.

1.2.2 Osmotic Stabilization and Protoplasts Regeneration

Protoplasts formed after treatment of mycelium with the abovementioned lytic enzymes need to be maintained in an osmotically stabilized lytic buffer, e.g., KCM (50 mM phosphate, pH 5.8, stabilized with 0.7 M KCl) (Cantoral et al. 1987). Other osmotic stabilizers, e.g., sucrose or some sugar alcohols (e.g., sorbitol) in concentration of 0.8–1.0 M have also been used. Concentrations of protoplasts (filtered through nylon cloth) in the range of $1–5 \times 10^7$ to 10^8 per transformation reaction (50–100 µL volume) are adequate. In many cases to achieve this number of protoplasts it will be necessary to concentrate the protoplasts by gentle centrifugation in osmotically stabilized buffer. The transformation process is mediated by polyethylene glycol (PEG) in presence of Ca^{2+} ions. One of the transformation solutions (PCM) uses 0.7 M KCl as osmotic stabilizer, 50 mM $CaCl_2$ and 10 mM MOPS buffer. The liquid PEG (MW 1,000–8,000) is mixed with the plasmid DNA in KCM buffer. PEG of higher molecular weight is too viscous or solid and is not adequate. Good transformation efficiencies are obtained with 25 % PEG in the transformation mixture and higher transformation efficiencies of *P. chrysogenum* are usually achieved by increasing the PEG concentration (up to 50 %). PEG is well known to cause fusion of protoplasts (Anné 1977). In the presence of Ca^{2+} ions the DNA is trapped and is introduced into the protoplasts, probably by PEG-induced endocytosis.

Regeneration of cell walls is carried out in complex medium with or without selection pressure. The direct regeneration in presence of the selective agent is adequate when a good transformation efficiency is routinely achieved. However, in some cases it is preferable to regenerate the transformed protoplasts in absence of the selective agent and then replicate the transformants in plates with the selective agents. When dealing with complementation of auxotrophic strains direct selection of the prototrophic transformants in minimal medium (e.g., Czapek medium) may be unfavorable for cell wall regeneration (J.M. Cantoral and J.F. Martín, unpublished results).

1.3 Alternative Transformation Procedures That Do Not Require Protoplasts

Although the PEG-assisted introduction of DNA in protoplasts is a good transformation procedure, several other methods that do not require protoplasts have been developed. Transformation of entire cells, assisted with lithium acetate (0.1 M) or with salts of other alkali metals, has been very successful in yeast (see Chap. 7 in this book) and has been reported in several filamentous fungi, including *N. crassa* (Dhawale and Marzluf 1985), *Coprinus cinereus* (Binninger et al. 1987), and *Ustilago violacea* (Bej and Perlin 1989). However, this method has not been widely used in filamentous fungi because of its limited transformation efficiency in these fungi.

Other alternative methods include (1) electroporation, (2) *Agrobacterium tumefaciens-mediated* transformation (de Groot et al. 1998), and (3) ballistic transformation (also named biolistic) (Ruiz-Diez 2002).

1.3.1 Electroporation

The electroporation of entire cells (or protoplasts) has been achieved successfully for several fungi. The use of cells (usually spores) avoids the need to obtain protoplasts. The spores of fungi may be pre-germinated to facilitate the electroporation (Ozeki et al. 1994; Chakraborty et al. 1991). Currently, electroporation is a reliable method for transformation of some well-known fungi but the protocols need to be optimized for each fungal species (Lakrod et al. 2003).

1.3.2 *Agrobacterium*-Mediated Transformation

It is well known that during plant infection *Agrobacterium tumefaciens* is able to transfer the T-DNA region of the Ti plasmid to the genome of the infected plant. The T-DNA region is bordered by two imperfect inverted repeats and it was found

that exogenous DNA inserted between the left and right borders of the T-DNA is also transferred during conjugation. The *A. tumefaciens* conjugation is mediated by the *vir* (virulence) genes (located in the Ti plasmid) products. These genes are induced by acetosyringone. The addition of this inducer is required for the successful transformation of fungal cells with this system. The T-DNA and the selectable markers are integrated at randon in the genome of the transformants.

The *Agrobacterium*-mediated transformation works well in *S. cerevisiae* (Bundock et al. 1995). Using *A. tumefaciens* plasmids containing the hygromycin-resistance marker de Groot et al. (1998) transformed *Aspergillus awamori* (Gouka et al. 1999). The *A. tumefaciens*-mediated conjugation was extended to several other fungi including the common ascomycetes, as *Aspergillus* (de Groot et al. 1998) and *Monascus purpureus* (Campoy et al. 2003), phytopathogenic ascomycetes (Malonek and Meinhardt 2001; Zwiers and de Waard 2001) and the basidiomycetes *Agaricus bisporus* (de Groot et al. 1998; Chen et al. 2000), *Hypholoma sublateritium* (Godio et al. 2004) and ectomycorrhizal fungi (Hanif et al. 2002).

The efficiency of transformation obtained with this method is similar to that with PEG-assisted transformation of protoplasts but the more difficult development of adequate binary vectors containing the *vir* genes and the heterologous DNA, and the need to optimize the *Agrobacterium*-fungi conjugation for each fungus, have limited its application.

1.3.3 Biolistic Transformation

Biolistic transformation using tungsten particles coated with DNA, which are introduced at high speed into fungal cells, is an alternative method to transform fungi which cannot be transformed by the other tools (Klein et al. 1987; Hazell et al. 2000). However, the required specialized equipment limits its utilization in many laboratories.

In a comparative study of four methods, namely transformation of protoplasts, *A. tumefaciens-mediated* transformation, electroporation, and biolistic transformation of *Aspergillus giganteus* with plasmid DNA carrying, in all cases, the same hygromycin-resistance marker, only the first two procedures were successful (Meyer et al. 2003).

1.4 Selective Markers

1.4.1 Nutritional Markers

Early success on transformation of filamentous fungi was achieved by several research groups in the mid-1980s in the UK, the USA, Spain, and other countries. They developed successful systems for transformation of *N. crassa, A. nidulans, A. niger, P. chrysogenum*, and a few other ascomycetes (Table 1.1) using different selective markers. The early success in the transformation of *N. crassa* was based on the use of the *pyr4* gene of this fungus to complement uracil auxotrophs (Buxton and Radford 1983). The same marker was then used to transform uracil auxotrophs of *A. nidulans* (Ballance and Turner 1985), and *P. chrysogenum* (Cantoral et al. 1987). The *trpC* marker was also used in early studies in *A. nidulans* (Yelton et al. 1984). Other authors used the acetamidase marker (Tilburn et al. 1983), or the *acuD* gene as nutritional markers that allowed the detection of transformants growing on acetamide (Beri and Turner 1987) or acetate-based media, respectively. The enzyme acetamidase encoded by the *amdS* gene is required for the utilization of acetamide as nitrogen source (Hynes 1979; Hynes et al. 1983).

The *amdS* gene was initially used to complement mutants of *A. nidulans* defective in this gene (Tilburn et al. 1983) but since acetamide is a poor nitrogen source for several *Aspergillus* species and allows very limited growth of the wild type strains, vectors carrying the *amdS* gene can be used as selectable markers to transform wild type strains because they confer faster growth to the transformants (Kelly and Hynes 1985, 1987). The same strategy may be used for other fungi but it requires a pre-study of their ability to grow on acetamide.

Table 1.1 Representative nutritional and resistance markers used in the initial studies on transformation of filamentous fungi

Transformed fungi	Marker gene	Origin of the gene	References
Neurospora crassa	*argB*	*Aspergillus nidulans*	Weiss et al. (1985)
Aspergillus nidulans	*argB*	*A. nidulans*	John and Peberdy (1984)
Aspergillus niger	*argB*	*A. nidulans*	Buxton et al. (1985)
Magnaporthe grisea, Nectria haematococca	*argB*	*A. nidulans*	Rambosek and Leach (1987)
Trichoderma reesei	*argB*	*A. nidulans*	Penttila et al. (1984)
Mucor circinelloides	*leuA*	*M. circinelloides*	Roncero et al. (1989)
Aspergillus niger, Aspergillus oryzae	*niaD*	*A. niger, A. oryzae*	Unkles et al. (1989a, b)
Penicillium chrysogenum	*niaD*	*A. nidulans*	Whitehead et al. (1989)
Fusarium oxysporum	*niaD*	*A. nidulans*	Malardier et al. (1989)
Neurospora crassa	*nic*	*N. crassa*	Akins and Lambowitz (1985)
Aspergillus nidulans	*pyr4*	*N. crassa*	Ballance et al. (1983)
Aspergillus flavus	*pyr4*	*N. crassa*	Woloshuk et al. (1989)
Penicillium chrysogenum	*pyr4, pyrG*	*N. crassa, P. chrysogenum*	Cantoral et al. (1987, 1988)
Aspergillus oryzae	*pyrG*	*A. oryzae*	Ruiter-Jacobs et al. (1989)
Neurospora crassa	*trp-1*	*N. crassa*	Schechtman and Yanofsky (1983)
Aspergillus nidulans	*trpC*	*A. nidulans, A. niger*	Yelton et al. (1984), Kos et al. (1985)
Penicillium chrysogenum	*trpC*	*P. chrysogenum*	Sánchez et al. (1987), Picknett et al. (1987)
Podospora anserina	*ura5*	*P. anserina*	Bégueret et al. (1984)
Aspergillus nidulans	*acuD*	*A. nidulans*	Ballance and Turner (1986)
Aspergillus niger, A. nidulans	*amdS*	*A. nidulans*	Kelly and Hynes (1985), Tilburn et al. (1983)
Cochliobolus heterostrophus	*amdS*	*A. nidulans*	Turgeon et al. (1985)
Penicillium chrysogenum	*amdS*	*A. nidulans*	Beri and Turner (1987)
Penicillium nalgiovense	amdS	*A. nidulans*	Geisen and Leistner (1989)
Trichoderma reesei	*amdS*	*A. nidulans*	Penttila et al. (1984)
Penicillium chrysogenum	*facA*	*P. chrysogenum*	Gouka et al. (1993)
Neurospora crassa	Benomyl^{R}	*N. crassa*	Orbach et al. (1986)
Penicillium chrysogenum	*ble*	*S. hindustanus*	Kolar et al. (1988), Casqueiro et al. (1999a, b)
Claviceps purpurea	*ble*	*S. hindostanus*	Van Engelenburg et al. (1989)
Tolypocladium geodes	*ble*	*S. hindostanus*	Calmels et al. (1991)
Aspergillus niger	G418	Tn5	Rambosek and Leach (1987)
Penicillium chrysogenum	G418	Tn5	Stahl et al. (1987)
Acremonium chrysogenum	G418	Tn903	Isogai et al. (1987)
Schizophyllum commune	G418	Tn5	Ulrich et al. (1985)
Aspergillus nidulans	*hygB*	*E. coli*	Punt et al. (1987)
Cochliobolus heterostrophus	*hygB*	*E. coli*	Yoder et al. (1986)
Acremonium chrysogenum	*hygB*	*E. coli*	Queener et al. (1985), Skatrud et al. (1987)
Fusarium oxysporum	*hygB*	*E. coli*	Kistler and Benny (1988)
Septoria nodorum	*hygB*	*E. coli*	Cooley et al. (1988)
Monascus purpureus	*aurA*	*A. nidulans*	Shimizu et al. (2006)
Penicillium chrysogenum	*Su*	R388 plasmid	Carramolino et al. (1989)
Penicillium chrysogenum	*oli*	*P. chrysogenum*	Bull et al. (1988)
Aspergillus nidulans	*oli*	*A. nidulans*	Ward et al. (1986)
Podospora anserina	*sen*	*P. anserina*	Tudzynski et al. (1980)
Penicillium chrysogenum, A. niger	tubulin	*A. niger*	Rambosek and Leach (1987)

1.4.2 Metabolic Fitness of Auxotrophic Host Strains: Growth and Secondary Metabolites Production

The use of auxotrophic mutants (e.g., *pyrG, argB, trpC*) or acetamidase-defective mutants in fungi has been important in the progress of fungal molecular biology. However, many of these mutations affect growth even when the strains are complemented with the adequate gene. In some cases (e.g., *pyrG*) the effect on growth rate is small (Díez et al. 1987) but in those procedures based on other nutritional markers, which may affect limiting steps in amino acids or vitamins biosynthetic pathways, the use of these markers may have a deleterious effect on growth (B. Díez, J.M. Cantoral and J.F. Martín, unpublished results).

These mutations might influence or even limit directly the biosynthesis of secondary metabolites [e.g., *acuD*, which encodes isocitrate lyase (Ballance and Turner 1986), an enzyme involved in the central pathways of precursors of polyketides]. Genes of the fungal lysine pathway that complement *lys* auxotrophs may be used as nutritional markers but they clearly affect β-lactam biosynthesis (Casqueiro et al. 1999a).

Another example is the use of the *facA* gene in a transformation system based on the complementation of mutants defective in acetate utilization (Gouka et al. 1993). The *facA* gene encodes an acetyl-CoA synthetase that catalyzes the activation of acetate units which are involved in the formation of polyketides and polyketide-nonribosomal peptide hybrid antibiotics.

A reduction of growth rate may have a negative effect on secondary metabolite volumetric production due to the reduced biomass in the cultures; however, in some cases a small reduction in protein or total RNA synthesis may be favorable for secondary metabolites biosynthesis because it saves NADPH, energy and precursors for secondary metabolite production. Indeed, expression of genes for secondary metabolite biosynthesis is usually higher at low specific growth rate, i.e., when the fungal cultures reach the end of the rapid growth phase. Despite this limitations in the industrial applications of auxotrophic strains, the complementation with adequate nutritional markers are very useful tools in fungal research.

1.4.3 Positive Selection Resistance Markers

A major drawback of the transformation strategies based on the complementation of auxotrophs is the need to obtain first the adequate auxotrophs. Furthermore, as indicated above, some of the auxotrophic mutations may affect growth. This problem was avoided by the introduction of dominant resistance markers (Table 1.1). One of the first examples of dominant resistance markers was the use of the resistance to the antifungic benomyl in *N. crassa* and *A. niger* (Table 1.1). The use of a hygromycin B-resistance marker was reported in early transformation of *A. chrysogenum* (Skatrud et al. 1987), *A. nidulans* (Punt et al. 1987), and *Cochliobolus heterostrophus*. Also the resistance to the aminoglycoside G418 was tested in *A. chrysogenum, P. chrysogenum*, and *A. niger* (Stahl et al. 1987; Rambosek and Leach 1987; Isogai et al. 1987). The marker of resistance to phleomycin used initially in *P. chrysogenum* and *Claviceps purpurea* has been widely utilized later (Durand et al. 1991; Casqueiro et al. 1999a, b; Bañuelos et al. 2001). This system, which used initially the phleomycin resistance gene from transposon Tn5 (Gatignol et al. 1987) and later from *Streptoalloteicus hindustanus* (Calmels et al. 1991) was commercialized by CAYLA (Toulouse, France) and is quite efficient for some filamentous fungi.

Another less frequently used selection markers are the resistance to oligomycin (*oli*) in *A. nidulans* and the resistance to sulfonamides (*su*) in *P. chrysogenum* (Table 1.1). The resistance to bialaphos and to the herbicide glyphosate has been tested but in general it is not very useful because high levels of those herbicides are required to inhibit the fungi.

A marker of resistance to aureobasidin has been used in *Monascus purpureus* by Japanese scientists (Shimizu et al. 2006). This antibiotic is expensive and the usefulness of this system in *P. chrysogenum* and *P. roqueforti* was limited.

Furthermore, a gene (*aurA*) which confers aureobasidin resistance has been found in *A. nidulans* (Kuroda et al. 1999) and it may be present in related fungi, what explains the low sensitivity of some ascomycetes to aureobasidin.

Once a resistance marker has been introduced in filamentous fungi it is difficult to remove it to obtain a "clean transformant" for a second round of genetic manipulations. Therefore, alternative markers have been explored. The *niaD* marker (encoding the nitrate reductase) is a good example of a nutritional marker that allows homologous complementation since *niaD* mutants are easily isolated and the complementing *niaD* gene is available from several fungal species (Gouka et al. 1991; Sánchez-Fernández et al. 1991). Spontaneous *niaD* mutants of *P. chrysogenum* and other fungi can be isolated by their inability to grow on media with nitrate as the only nitrogen source, although some of them are unstable. Stable *niaD* mutants are enriched by selecting clones resistant to chlorate which lack completely nitrate reductase. These mutants are routinely complemented with vectors carrying the homologous *niaD* gene, which integrates mostly into nonhomologous DNA regions (Gouka et al. 1991). The nitrate reductase gene of *A. nidulans* was used to transform a chlorate-resistant mutant of *Penicillium caseicolum* (Daboussi et al. 1989). This system may be useful for self-cloning of homologous DNA in fungi used in food processing or ripening since it does not involve antibiotic resistance genes.

Similarly *pyrG* (*pyr4* in *Neurospora*) mutants are easily selected by resistance to the toxic antimetabolite 5-fluoroorotic acid (5-FOA) (Díez et al. 1987). The *pyrG* mutants are easily complemented by the *pyrG* gene of the same fungus thus allowing self-cloning procedures.

1.5 Autonomously Replicating Plasmids: Stability Problems of Gene Libraries

Autonomously replicating plasmids which are maintained in a non-integrated form are interesting tools for some genetic studies. Yeast autonomously replicating plasmids (YRp) and episomal (YEp) vectors developed in *S. cerevisiae* (Stinchcomb et al. 1979) are based on the ARS (*a*utonomously *r*eplicating *s*equences) of chromosomes or 2μ yeast extrachromosomal element (Gasser 1991). Transformation of *P. chrysogenum* (Cantoral et al. 1987) and other ascomycetes with YRp10 was attempted but it was unsuccessful, indicating that yeast DNA replication origins do not work in filamentous fungi.

A transformation enhancing sequence was cloned from an *A. nidulans* genomic library and named AMA1 (Gems et al. 1991; Gems and Clutterbuck 1993). Plasmids containing the AMA1 sequence increased the efficiency of transformation of *A. nidulans* by 1,000- to 2,000-fold and were shown to replicate autonomously (Aleksenko and Clutterbuck 1996, 1997).

The AMA1 nucleotide sequence shows a 2.2 kb duplicated sequence in opposite orientations separated by a unique 0.6 kb central region (Fig. 1.1). There are no long ORFs in the AMA1 sequence, indicating that it does not encode large polypeptides (Aleksenko et al. 1995). The repeated sequence is present in single copies in the genome of *A. nidulans* (chromosome III) and belongs to the MATE (*M*obile *Aspergillus t*ransformation *e*nhancers) family. When introduced on plasmids containing either a nutritional marker (e.g., *pyrG*) or a dominant resistance marker (e.g., *ble*), the AMA1 sequence drastically increases the transformation efficiency in *P. chrysogenum* (Fierro et al. 1996). These authors constructed a series of plasmids containing fragments of AMA1 (pAMPF2 to pAMPF12, and pAMPF21) that allowed to study the role of each of the repeated sequences and the central region in the transformation (Fig. 1.1). Each of the 2.2 kb repeats was sufficient to obtain high transformation efficiency. Deletion of the 0.6 kb central region between the two arms has no significant effect on transformation efficiency, but when the 2.2 kb arms (each of them) were trimmed down, the remaining fragments were clearly less efficient (Fierro et al. 1996).

In summary, at least one of the 2.2 kb repeated regions is required and sufficient for optimal transformation. Studies on the fate of the transformed

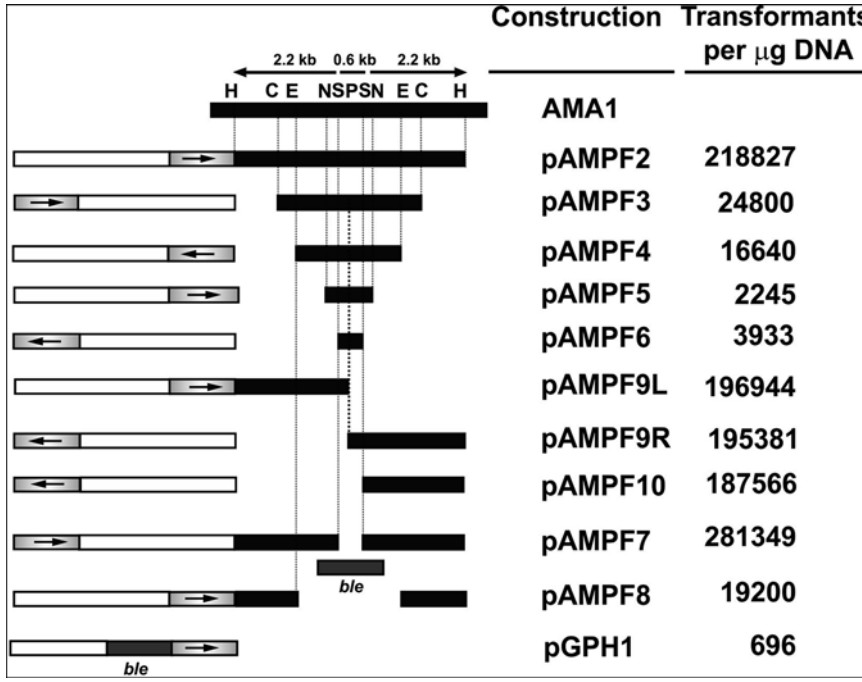

Fig. 1.1 The autonomously replicating sequence AMA1 of *Aspergillus nidulans* and vectors derived from it. The AMA1 region is shown in *black* and the *pyrG* gene in shaded *gray* with an *arrow* indicating the orientation of the gene. Two vectors carry, in addition, the *ble* gene (indicated in *dark gray*). The two 2.2 kb arms and the 0.6 kb central region of AMA1 are shown by *arrows* and a *thin line*, respectively. The number of transformants to prototrophy obtained by complementing *P. chrysogenum nep6 pyrG* is shown on the right. Drawn with data of Fierro et al. (1996) and Aleksenko and Clutterbuck (1996). The main restriction sites in the AMA1 sequence are indicated by *capital letters*

DNA showed that the AMA1-containing plasmids replicate autonomously in the nucleus and does not integrate in the chromosomes of the transformants (Fierro et al. 1996), although sometimes limited integration has been reported in *A. nidulans* (Aleksenko and Clutterbuck 1997). The autonomously replicating plasmids are unaltered in the transformants but they may form multimers, resulting in a high molecular weight DNA. In *A. nidulans* and *P. chrysogenum,* plasmids containing the full AMA1 sequence appear to be mitotically stable (Fierro et al. 1996; Aleksenko et al. 1995). In addition to *A. nidulans* and *P. chrysogenum*, AMA1-containing vectors have been used to transform *A. parasiticus* (Moreno et al. 1994), *Zalerion arboricola* (Kelly et al. 1994), *P. nalgiovense* (Fierro et al. 2004), *Gibberella fujikuroi* (Brückner et al. 1992), and *P. canescens* (Aleksenko et al. 1995).

Due to their high transformation efficiency AMA1-containing vectors are good systems to construct libraries and to clone genes. However, autonomous replicating plasmids carrying fragments of chromosomal DNA from fungal libraries are highly recombinogenic in *E. coli*. When a library of *P. chrysogenum* DNA fragments in the AMA1-containing pAMPF9L plasmid was transformed into *Penicillium* to study their effect on penicillin biosynthesis, and the recombinant plasmids that enhance antibiotic production were rescued in *E. coli*, we obtained many rearranged sequences in the transformants; this limits the use of AMA1-containing plasmids for direct cloning strategies (B. Díez, J.L. Barredo and J.F. Martín, unpublished results).

The presence of ARS in some DNA fragments of *Mucor circinelloides* has been reported. These sequences were cloned when complementing *leu*

autotrophic mutants of *M. circinelloides* (Roncero et al. 1989) and methionine auxotroph mutants (Anaya and Roncero 1991). These *M. circinelloides* plasmids do not replicate in *S. cerevisiae* and the role of the cloned ARS elements that complement distinct auxotrophies is intriguing.

1.6 Homologous and Nonhomologous Recombination: Integration of Exogenous Genes

Targeted integration of exogenous DNA is a valuable tool for disruption or modification of genes of interest in fungi. From the results of the early transformation experiments it was evident that integration of vectors in the fungal genome was frequently ectopic (Ballance et al. 1983; Ballance and Turner 1985; Walz and Kuck 1993; Cantoral et al. 1987). Even when the vector harbors a homologous gene, integration frequently takes place via nonhomologous recombination in contrast to *S. cerevisiae* which is highly efficient in homologous recombination. As few as 4 bp in the homologous DNA fragment were reported to be sufficient to allow homologous recombination in this yeast (Schiestl and Petes 1991). On the other hand in the filamentous fungi the efficiency of homologous recombination varies greatly depending on the fungus. In general the efficiencies of homologous recombination are enhanced by increasing the size of the homologous DNA fragment flanking the gene of interest (Casqueiro et al. 1999a, b), ranging from 4 % when the homologous DNA fragment is 1 kb in *A. nidulans* (van den Homberg et al. 1996) to 15 % with a homologous DNA fragment of 0.5 kb in *Glomerella cingulata* (Bowen et al. 1995) and 82 % with a homologous region of 3.1 kb in *Alternaria alternata* (Shiotani and Tsuge 1995). In *P. chrysogenum* the homologous recombination efficiencies were lower; in a study on the directed integration at the *lys2* locus of *P. chrysogenum* we found 1.6 % disruption events with a homologous DNA fragment of 4.9 kb. Experiments on double crossover at the same locus showed an efficiency of 0.14 % with a similar homologous DNA fragment (Casqueiro et al. 1999a).

The efficiency of recombination is also affected by the locus that is targeted, e.g., in *A. nidulans* targeted integration at the *niaD* locus is at least five times more effective than targeting at the *amdS* locus (Bird and Bradshaw 1997). Another factor that affects the frequency of homologous recombination is the topology of the DNA in the transforming plasmid. Frequently, linearized plasmid DNA is used for the recombination since studies in *S. cerevisiae* showed that double strand breaks increase the recombination efficiency (Orr-Weaver et al. 1981); the same occurs in *A. alternata* (Shiotani and Tsuge 1995), although in other fungi the linearization of plasmid DNA appears to have no influence (Walz and Kuck 1993; Hoskins et al. 1990; Dhawale and Marzluf 1985). Finally, the position of the homologous stretch of DNA with respect to the ends (double strand breaks) of the linearized plasmid (ends-in versus ends-out) may also affect the efficiency of homologous recombination.

1.6.1 Targeted Monocopy Integrations at Specific Loci

Due to the frequent ectopic integration of the transforming DNA it is difficult to obtain reliable data on the expression of exogenous genes after introduction in host strains, since their degree of expression is greatly influenced by the locus of ectopic integration. Indeed, it is well known that epigenetic factors related to the chromatin structure have a profound influence on gene expression in fungi (Bok et al. 2006; Shwab et al. 2007). The chromatin structure is affected by the product of genes such as *laeA* and *veA*. The LaeA protein is a nuclear protein with a methyltransferase domain that in association with other proteins appears to rearrange the chromatin structure (Bok et al. 2006; Kosalková et al. 2009).

Furthermore, the overall expression of integrated genes is also affected by the number of copies integrated in a genome (additive expression). To avoid these problems when quantifying the expression from a promoter of interest coupled to a reporter gene (e.g., *lacZ*) or by qRT-PCR, we used directed integration at a specific locus of all the constructs, and selected single

copy recombinants. Vectors containing the *pyrG* gene together with the promoter-reporter gene of interest were used (Gutiérrez et al. 1999). The constructs were targeted to the *pyrG* locus in a host strain containing a *pyrG* point mutation (Díez et al. 1987; Bañuelos et al. 2001). The integration at this locus is easily detected because it rescues the *pyrG* auxotrophy. Monocopy integrants at the *pyrG* locus are identified by their known restriction pattern that allows to distinguish single copy from multicopy transformants. This strategy has been successfully used with different promoters (Gutiérrez et al. 1999; Kosalková et al. 2000).

The monocopy integration strategy has been utilized to quantify carbon catabolite regulation, pH-regulation and activation by the PTA1 transcriptional enhancer of the *pcbAB* and *pcbC* genes of *P. chrysogenum* (Gutiérrez et al. 1999; Kosalková et al. 2000, 2007). Directed integration at specific loci may be particularly useful to achieve high expression if the integration site is located in DNA regions in which genes are highly expressed as occurs in certain chromosome regions (Palmer and Keller 2010).

1.6.2 The "Two Markers" Selection Strategy for Gene Disruption

Since the transformation efficiency of most filamentous fungi is low and integration occurs largely at nonhomologous sites in the genome (ectopic integration), it was difficult to obtain directed integration to inactivate specific genes of interest (gene X in Fig. 1.2). This problem was solved by the use of the "two markers" strategy. This approach is based on the use of homologous (preferably identical) sequences flanking the gene of interest. A construction is made in which the flanking sequences (1–5 kb) are linked to a resistance gene to achieve a replacement of the chromosomal gene of interest by this first resistance "marker A" by double crossover. As the frequency of double crossover is usually very low a second selective "marker B" is introduced in the transforming plasmid construction (Fig. 1.2). Since the double crossover results usually in the loss of the carrier plasmid, the selection of transformants that have integrated the "marker A" (i.e., the gene replaced) and lost the second plasmid-borne marker B greatly facilitates to isolate the correct transformants. This strategy has been very successfully used to knockout several genes in *P. chrysogenum* (Liu et al. 2001) and *A. chrysogenum* (Ullan et al. 2002).

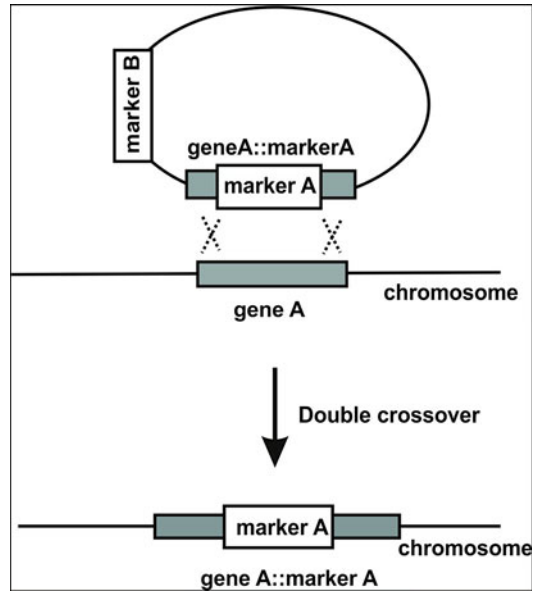

Fig. 1.2 Model of the "two markers" system for targeted integration and selection of transformants. Integration is targeted at the "gene X" locus by two homologous DNA fragments of gene X bordering the "marker A" gene. After crossover, the "X gene" is replaced by "marker A." The second marker (B in the transforming vector) is lost during recombination, thus allowing an easy selection of recombinants. (Modified from Liu, G. and J. Casqueiro, O. Bañuelos, R.E. Cardoza, S. Gutiérrez, and J.F. Martín. 2001. Targeted inactivation of the *mecB* gene, encoding cystathionine-gamma-lyase, shows that the reverse transsulfuration pathway is required for high-level cephalosporin biosynthesis in *Acremonium chrysogenum* C10 but not for methionine induction of the cephalosporin genes. J. Bacteriol. 183:1765–1772 with permission)

1.6.3 The Nonhomologous End-Joining Mechanism

The ectopic integration is mediated by the nonhomologous end joining (NHEJ) system that was found in humans (Pastwa and Błasiak 2003) and fungi (Ninomiya et al. 2004). NHEJ is performed by the catalytic subunit (DNA-PKcs of the NHEJ complex) that is targeted to the nonhomologous

sequences where integration occurs in the genome by a complex of two proteins, Ku70 and Ku80. Additional enzymes (DNA ligase IV and the protein Xrcc4) complete the DNA repair, following the integration.

Due to the interest in decreasing nonhomologous recombination, the NHEJ system has been investigated in some non-conventional yeasts (e.g., *Kluyveromyces lactis* (Kooistra et al. 2004)) and several filamentous fungi, including *Neurospora crassa* (Ninomiya et al. 2004; Ishibashi et al. 2006), *Claviceps purpurea* (Haarmann et al. 2008), several aspergilli [*A. nidulans* (Nayak et al. 2006), *A. niger* (Meyer et al. 2007), *A. fumigatus* (Krappmann et al. 2006), *A. oryzae* and *A. soyae* (Takahashi et al. 2006), and *A. parasiticus* (Chang 2008)], and *P. chrysogenum* (Snoek et al. 2009). Mutants defective in the targeting proteins Ku70 and/or Ku80 have been isolated for each of these fungi. Although in general the overall transformation efficiency was not increased or even reduced in some of these mutants, the percentages of nonhomologous recombination was greatly reduced in all of them and therefore, the use of *ku-orthologue* mutants of fungi as host strains allows easy selection of recombinants at homologous sites. The proportion of correct homologous recombinants obtained varied from 100 % in *K. lactis* and *N. crassa* to 60 % in *Claviceps purpurea* and 47–56 % in *P. chrysogenum* (Snoek et al. 2009). These are impressive efficiencies as compared to about 1 % homologous recombination in the control (non-mutated) *P. chrysogenum* strain.

The nonhomologous recombination-defective mutant still require a stretch of homologous DNA flanking the targeting gene, for high frequency of homologous recombination. This ranges from 0.5 kb in *A. nidulans* (Nayak et al. 2006; Meyer et al. 2007) to 1 kb in *P. chrysogenum* and *N. crassa* (Snoek et al. 2009; Krappmann et al. 2006).

1.6.4 Metabolic Fitness of NHEJ-Deficient Mutants

An important question is the genetic stability (and indirectly the metabolic fitness) of the NHEJ-defective mutants. Since the system is involved in DNA damage (strand break) repair, fungal mutants defective in the nonhomologous recombination system are prone to increased sensitivity to a variety of mutagens, including radiations (e.g., X-rays and UV-light) and chemical mutagenic agents such as methylmethanesulfonate or bleomycin (a compound that induces double strand breaks), among others. Indeed, *ku-orthologue* mutants of *N. crassa* have been reported to be sensitive to methylmethanesulfonate and bleomycin (Ishibashi et al. 2006). Mutants of *A. niger* are sensitive to X-rays and UV-light (Meyer et al. 2007). However, in *nku* mutants (impaired in the *ku* orthologue) of other fungi no sensitivity changes were observed.

The apparent lack of pleotropic effects on fungal metabolism in Ku70 and Ku80 mutants has stimulated their widespread use. However, in a comparative transcriptome analysis of a mutant in the *ku70*-like gene (named *P. chrysogenum* Δ*hdfA*) and the control (parent) strain, a small number of genes with altered expression were identified. Most of these encode proteins of unknown function but three were putative transporters of glucose or other carbohydrates (Snoek et al. 2009), that may affect the growth rate of such mutants.

Alternatively, the gene encoding DNA ligase IV that is involved in the last step of the nonhomologous recombination system has been disrupted in *N. crassa* and *A. oryzae* (Ishibashi et al. 2006; Takahashi et al. 2006). This mutation also greatly increased the frequency of homologous recombination (Maruyama and Kitamoto 2008). However, the possible side effects of disrupting this DNA ligase has not been fully studied.

The relevance of the sensitivity of mutants defective in the NHEJ system to mutagenic radiations and chemical agents is still obscure. However, it is likely that some environmental radiations and chemical compounds in the culture media will affect these strains, and therefore their usefulness for metabolic engineering of the production of secondary metabolites. This problem may be solved by transient (temporal) removal of the NHEJ system to facilitate obtention of the desired transformants, followed by reintroduction of the deleted ku-70/ku-80 gene to avoid the problems derived from its removal (Nielsen et al. 2008).

1.7 Future Outlook

The transformation tools available during the last 50 years have steered the progress in the molecular biology of fungi. Currently, more than 100 fungal species have been transformed efficiently and some model fungi have been studied in great detail. However, much more research effort is needed to obtain reliable and efficient transformation procedures for rare fungi. Vectors and transformation protocols of some basidiomycetes and zygomycetes need to be improved. Targeted gene disruption and controlled gene expression, together with basic information derived from genomic, proteomic, and transcriptomic studies will lead to comprehensive functional gene analysis and will allow the development of engineered strains of importance for our society. For this purpose the knowledge on the mechanisms that control gene expression need to be fully understood. Finally the integration of biochemistry, molecular genetics, and molecular ecology of fungi will provide a comprehensive view on fungal systems biology.

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Part II

Transformation Methods: Protoplast Transformation

Protoplast Transformation for Genome Manipulation in Fungi

2

Aroa Rodriguez-Iglesias and Monika Schmoll

2.1 Introduction

Fungi have gained attention in recent years for their importance in health and economy. Acting in symbiotic relationships with plants as biofungicides, producing numerous drugs as antibiotics and hydrolytic enzymes, for example, for second generation biofuels, and causing plant and animal diseases, the importance of fungi is remarkable nowadays (Borkovich and Ebbole 2010). Furthermore, the increase of available sequenced genomes of different fungi allows functional genomic analyses to elucidate mechanisms of gene regulation for enzyme production, substrate sensing, signaling cascades, etc. as well as their interconnections and conserved metabolic pathways in different fungi. Available molecular genetic tools allow the transfer of DNA to a targeted locus in the genome (Olmedo-Monfil et al. 2004). Combinations of these tools with different transformation systems are being used for efficient genetic modifications of fungi for research purposes and industrial strain improvement. High throughput approaches have been developed to facilitate construction of whole genome knockout libraries, for example, for *Neurospora crassa* (Collopy et al. 2010) or *Trichoderma reesei* (Schuster et al. 2012).

Protoplasting is one of the methods commonly used to prepare cells in order to genetically modify fungi. More than six decades have passed since the first reports appeared on protoplast isolation from yeasts (Bachmann and Bonner 1959; Eddy and Williamson 1959) and filamentous fungi (Bachmann and Bonner 1959; Fawcett et al. 1973). Since then, the interest in the improvement of this technique has enabled scientists to achieve higher transformation rates and more efficient targeting to the appropriate genomic locus. Protoplast preparation requires removal of the fungal cell wall, nowadays predominantly by using enzymes. Mechanical and other nonenzymatic methods have been reported, but they have not been used extensively due to their inconvenience. Nevertheless, some of these disadvantages could be due to the specific procedure for each particular organism and the physiological state that might be induced in the protoplasts as a consequence of the treatments used (Sun et al. 1992; Von Klercker 1982). Alternative methods to protoplast transformation include electroporation, biolistics, and *Agrobacterium* transformation (see other chapters of this book), which were established to improve the transformation efficiency for some species that are not suitable for protoplast transformation. The simplicity in technical operations and materials required for this method make the protoplast transformation the most commonly

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selected method to perform transformations in filamentous fungi. Therefore, enzymatic methods for protoplasting have early on become the choice for most labs (Peberdy 1979). However, with the recent high throughput projects for gene manipulation, electroporation is increasingly applied (Park et al. 2011; Schuster et al. 2012).

In this chapter we provide an overview about different steps of genetic transformation of fungi and a review of different protocols for protoplast transformation in fungi.

2.2 The Fungal Cell Wall: A Barrier to be Removed

The first obstacle found when intending to transfer exogenous DNA into the fungal cell is the cell wall. Most protoplasting procedures remove this cell wall by using enzymatic digestion, mainly using fungal enzymes (Gruber and Seidl-Seiboth 2012). Therefore, the structure of fungi cell wall determines the enzymes needed to digest it (Adams 2004).

2.2.1 Composition of the Fungal Cell Wall

The main components of the fungal cell wall are chitin, 1,3-β- and 1,6-β-glucans, proteins, mannans, and other polymers, which are cross-linked forming this complex structure. The crystalline polysaccharides, chitin and β-glucans, constitute the skeletal portion of the wall, whereas the amorphous polysaccharides and protein–polysaccharide complexes are components of the wall matrix. The shape, integrity, and mechanical strength of the fungus is determined by the chemical composition and the cell wall, which is responsible for the interaction of the fungus with its environment as well as biological activity (Adams 2004; Gooday 1995; Lesage and Bussey 2006).

For most of fungi, the central core of the cell wall is a branched β-1,3/1,6-glucan that is linked to chitin via a β-1,4 linkage. β-1,6 glucosidic linkages between chains account for 3 % and 4 % of the total glucan linkages, respectively, in *Saccharomyces cerevisiae* and *A. fumigatus* (Adams 2004; Fleet 1991; Fontaine et al. 2000; Gooday 1995; Kollar et al. 1995; Lesage and Bussey 2006; Perez and Ribas 2004). The cell wall structures of these fungi were investigated in detail. This structural core, which determines the mechanical strength, is suggested to be fibrillar and embedded in an amorphous cement (usually removed by alkali treatment) (Latgé 2007). This structure varies markedly between species of fungi, although there is a close correlation between taxonomic classification and cell wall composition among fungi determined by their evolution (Bartnicki-Garcia 1968; Borkovich and Ebbole 2010). The basic composition of all fungal cell walls, including those of the distant chytrids, consists of a branched polysaccharide of β-1,3/1,6 glucan that is linked to chitin via a β-1,4 linkage. This ancient ancestral fungal cell wall has been further modified and decorated in the various fungal orders (Borkovich and Ebbole 2010). As an example, α-l,3 glucan is present in cell walls of Ascomycetes and Basidiomycetes. Following their bifurcation, β-1,6 glucan was added to Ascomycetes and xylose to Basidiomycetes (Kollar et al. 1997; Ruiz-Herrera et al. 1996; Vaishnav et al. 1998). Within the Ascomycetes, more differences have been developed between the yeasts and filamentous fungi. The yeast *S. cerevisiae* contains β-1,6 glucan, whereas filamentous fungi such as *A. fumigatus* contain linear α-1,3/1,4 glucan and galactomannan with galactofuran side chains (Fontaine et al. 2000; Kollar et al. 1997). Regarding to chitin content, cell walls of filamentous fungi contain higher levels than those of yeasts (approximately 15 % vs. 2–3 %, respectively). The reason may be that the cell wall of filamentous fungi is cylindrical and under high turgor pressure, therefore it needs to increase its rigidity (Borkovich and Ebbole 2010). The attachment to embedded proteins and mannans also differs between filamentous fungal cell walls from those of the yeasts. Mannan chains in *S. cerevisiae* cell wall are only found attached to cell wall proteins (CWPs), whereas in *A. fumigatus*, mannan chains are also found directly linked to glucans (Borkovich and Ebbole 2010; Latgé 2007; Lesage and Bussey 2006).

These differences in the cell wall composition between yeast and filamentous fungi may have appeared because of different evolutionary pressures. The adaptation of the filamentous fungal cell wall to extremely rapid deposition and growth at the hyphal tip and its ability to penetrate hard surfaces (Collinge and Trinci 1974) differ from the isotropic growth usually confined to surfaces in yeast (Borkovich and Ebbole 2010). This could be the reason why mutations in secretory and transport-related genes in yeast usually result in less severe phenotypic consequences than in filamentous fungi (Borkovich and Ebbole 2010). The morphological complexity of filamentous fungi makes slight disturbances in homeostasis more obvious than they are in yeast (Seiler and Plamann 2003; Whittaker et al. 1999).

2.2.2 Functions and Biological Activity of the Fungal Cell Wall

Concerning the biological activity of fungal cell wall, it provides protective and aggressive functions. It acts as an initial barrier in contact with hostile environments. Its absence can cause the death of the fungus unless there is an osmotic stabilizer. Regarding to the aggressive function, it harbors hydrolytic and toxic molecules, required for a fungus to invade its ecological niche. Furthermore, its rigid structure is useful as a force for the penetration of insoluble substrates that it colonizes or invades (Latgé 2007).

Polysaccharides, which represent 80–90 % of the dry matter of fungal cell walls, include amino sugars, hexoses, hexuronic acids, methylpentoses, and pentoses (Bartnicki-Garcia 1970). Glucose and *N*-acetyl-D-glucosamine (GlcNAc) usually represent the chemical elements of skeletal wall polysaccharides, such as chitin, noncellulosic β-glucans, and a-glucans.

Each component of cell wall performs specific functions, owing to distinctive physicochemical properties. Chitin and β-glucans are responsible for the mechanical strength of the wall, while the amorphous homo- and heteropolysaccharides, often in association with proteins, act as cementing substances and constitute the carbohydrate moieties of extracellular enzymes and cell wall antigens (Ballou and Raschke 1974; Gander 1974; Lampen 1968). The location within of cell wall components the wall structure also plays a role in the functional specialization. The outer surface of the cell wall is composed of amorphous material (often glycoprotein in nature), leading to a smooth or slightly granular texture. The interfibrillar spaces of the inner wall layer are filled with amorphous material, similar to that of the outer wall layer. In contrast, the layer of the wall adjacent to the plasmalemma is mainly composed by skeletal microcrystalline components. Due to plasticity of fungal cell walls, the structure varies during different growth phases. Newly synthesized portions of the walls are thin and smooth, whereas older portions have the primary wall covered with secondary layers composed of amorphous matrix material (Hunsley 1973; Hunsley and Gooday 1974; Hunsley and Kay 1976; Trinci 1978; Trinci and Coolinge 1975). Consequently, young mycelium is more vulnerable to enzymatic degradation than aged hyphae.

2.2.2.1 Chitin

Chitin, an α,β-(1,4) polymer of *N*-acetylglucosamine (GlcNAc), is the second main cell wall fiber (Borkovich and Ebbole 2010). It forms microfibrils that are stabilized by hydrogen bonds. Chitin provides tensile strength to the cell wall and composes approximately 2 % of the total cell wall dry weight in yeast, and 10–15 % in filamentous fungi (Borkovich and Ebbole 2010; Klis et al. 2002; Roncero 2002), even though previous literature reports that chitin represents between 0.3 and 40 % of the filamentous fungi cell wall dry weight (Mol and Wessels 1990; Molano et al. 1980; Sietsma and Wessels 1990). The absence of chitin and most glucans from plant and mammalian species makes these components of the fungal cell wall potential and actual targets for antifungal drugs (Beauvais and Latgé 2001; Gooday 1977; Latgé 2007; Nimrichter et al. 2005; Selitrennikoff and Nakata 2003). Synthesis of chitin is well studied in yeast and occurs mainly in the plasmalemma (Braun and Calderone 1978; Jan 1974), specifically,

at sites of polarized growth (Lenardon et al. 2010). Depending on the growth phase, chitin synthesis can occur at the bud tip (early bud growth) (Sheu et al. 2000) or over the entire bud surface (isotropic growth) in *S. cerevisiae*. After nuclear division, chitin is directed towards the mother-bud neck to prepare for cytokinesis (Lenardon et al. 2010).

Chitin is folded to form antiparallel chains, attached through intra-chain hydrogen bonds that lead to formation of strong fibrous microfibrils (Lenardon et al. 2010). In the zygomycetes the polymer chitosan is produced when part of the chitin is deacetylated immediately after synthesis but before chains crystallize by one or more chitin deacetylases (Ariko and Ito 1975; Davis and Bartnicki-Garcia 1984). However, in *C. albicans* less than 5 % of chitin is deacetylated to chitosan, while zygomycetes and the basidiomycete *Cryptococcus neoformans* have more than two-thirds deacetylated to chitosan (Baker et al. 2007; Bartnicki-Garcia 1968). Chitosan could be more elastic and resistant to the action of hostile chitinases than chitin (Lenardon et al. 2010). Nevertheless, partially deacetylated glucosaminoglycans are also observed (Datema et al. 1977b). Through ionic interactions, these insoluble polycationic polymers bind to essentially soluble polyanionic glycuronans (containing glucuronic acid, fucose, mannose, and galactose), which are maintained insoluble in the wall (Datema et al. 1977a, b). In contrast, the chitin (glucosaminoglycan) of the walls of ascomycetes and basidiomycetes is fully acetylated and associated with (1-3)-β-/(1-6)-β-glucan in an alkali-insoluble complex (Wessels 1994).

2.2.2.2 Glucans

Glucans, D-glucose polymers, represent the main fibrous component of the cell wall (Borkovich and Ebbole 2010). Glucans differ both in type and in relative proportions of individual glycosidic bonds. β-glucans are most abundant in fungal cell walls, present usually as constituents of the skeletal microfibrillar portions of the walls, although some fungi also contain glucose polymers linked by α-glycosidic bonds, such as *S. pombe* (Hochstenbach et al. 1998). Mainly, glucans can be found in long β-1,3 or short β-1,6-linked chains forming the main bulk (30–60 %, dry weight) of the cell wall (Borkovich and Ebbole 2010). β-1,3 glucans have a coiled spring-like structure that confers elasticity and tensile strength to the cell wall (Borkovich and Ebbole 2010). β-1,6 glucan acts as a flexible glue by forming covalent cross-links to β-1,3 glucan and chitin and to cell wall mannoproteins (Kapteyn et al. 2000; Kollar et al. 1997; Lowman et al. 2003; Shahinian and Bussey 2000; Sugawara et al. 2004). α-1,3 glucan harbors an amorphous structure and forms an alkali-soluble cement within the β glucan and chitin fibrils (Beauvais et al. 2005; Grün et al. 2005).

Synthesis of glucans almost exclusively occurs in the cell wall or at the outer surface of the plasmalemma, due to their insolubility and high degree of crystallinity (Farkas 1979). Furthermore, the fungal cell wall contains abundant branched 1,3-β- and 1,6-β-cross-links between proteins and homo- and heteropolysaccharides, chitin, glucan, and other wall components (Cabib et al. 2001; Klis et al. 2006). Nevertheless, the linkages between the individual macromolecular components of the wall do not involve ligase-type enzymes. There is a self-assembly of subunits in the formation of wall fabric through physicochemical interactions, apart from the non-catalytic formation of disulfide bridges between the protein moieties of wall glycoproteins. The number of chemical and physicochemical links increases with the cell age. Hence, older portions of the walls are more resistant to attacks by endogenous (Polacheck and Rosenberger 1975) as well as exogenous (Brown 1971; Necas 1971; Villanueva 1966) polysaccharide hydrolases. Therefore, protocols to remove fungal cell walls require young cells—generally between 16 and 20 h after germination—for protoplast preparation. Changes during germination vary depending on the fungus. For example, *P. notatum* spores increase the cell wall content of glucosamine, galactosamine, and glucose during the transition from resting spores to swollen spores, to germlings, and to grown mycelium, while galactose content is decreased when spores reached the swollen stage (Martin et al. 1973).

However, *Colletotrichum lagenarium* conidia show a decrease in the content of mannose in the cell wall, and xylose and rhamnose disappear from the mycelial wall (Auriol 1974). Surprisingly, it was reported that in *T. viride*, the cell wall from conidia does not contain chitin, in contrast to grown mycelium (Benitez et al. 1976). The fungus *Puccinia graminis* var. *tritici* was shown to undergo less changes during germination, such as decreasing the amounts of neutral sugars in the cell walls and increasing the content of chitin and protein in the mycelial wall (Ellis and Griffiths 1974).

2.2.2.3 Non-cellulosic β-Glucans

(1,3;1,4)-β-D-Glucans consist of unbranched and unsubstituted chains of (1,3)- and (1,4)-β-glucosyl residues. The physicochemical properties of the polysaccharides and the functional properties in cell walls depend on the ratio of (1,4)-β-d-glucosyl residues to (1,3)-β-D-glucosyl residues (Burton and Fincher 2009). These polysaccharides are not extensively found in fungi, only in certain species as the pathogenic fungi *Rhynchosporium secalis* (Pettolino et al. 2009). The regular distribution of polysaccharides in the cell wall due to the molecular links formed by (1,3)-β-glucosyl residues results in less soluble and less suitable gel formation in the matrix phase of the wall. The gel-like material offers some structural support for the wall, combined with flexibility and porosity. Moreover, these interactions can be influenced by associations with other polysaccharides or proteins in the cell wall (Burton and Fincher 2009).

2.2.2.4 Mannans

Mannans are polymers of mannose and can be found as α-1,6/α-1,2 or α-1.3/β-1,2 mannan chains either attached directly to glucans or covalently attached to proteins via asparagine (N-linked) or serine/threonine (O-linked) amino acid residues (Cutler 2001; Shibata et al. 2007). They comprise 10–20 % of the dry weight of the cell wall (Borkovich and Ebbole 2010; Lesage and Bussey 2006).

2.2.2.5 Proteins

Polysaccharide–protein complexes represent a principal cell wall constituent, especially in yeasts (Bartnicki-Garcia 1968; Calderone and Braun 1991; Phaff 1971). The amount of glycoproteins in the cell wall is variable, between 10 and 40 %, whereas the actual polypeptide content is about 4 % (de Groot et al. 2007; Klis et al. 2002). The main role of proteins in the wall is to modify and crosslink the wall polymers, instead of being involved in structural maintenance. Moreover, proteins exposed to the outer surface can also participate in determining antigenic and adhesive properties (Calderone and Braun 1991; Hazen 1990).

2.2.3 Enzymes, Biosynthesis, and Degradation of the Fungal Cell Wall

The structure of the fungal cell wall is highly dynamic and changes constantly during cell expansion and division in yeast, growth, morphogenesis, spore germination, hyphal branching, and septum formation in filamentous fungi. Hydrolytic enzymes, which are closely associated with the cell wall, are responsible for the maintenance of wall plasticity and functions during mycoparasitism (Gruber and Seidl-Seiboth 2012). Among hydrolases identified to date, chitinases, glucanases, and transglycosylases are found to be responsible for breaking and reforming of bonds within and between polymers, leading to re-modeling of the cell wall during growth and morphogenesis (Adams 2004; Lesage and Bussey 2006). The presence of these polysaccharide hydrolases in fungi indicates an autolytic activity in the fungal cell wall (Barras 1972; Fevre 1977; Fleet and Phaff 1974). This autolytic function involves not only cell wall weakening during growth and other morphogenetic processes (Adams 2004; Lesage and Bussey 2006). Therefore, the cell wall of fungi is a dynamic structure whose functions may vary with environmental conditions and in the course of cell and life cycles (Farkas 1979).

Composition of the cell wall determines the enzymes required to digest this structure. Therefore, removal of the fungal cell wall composed by glucans or cellulose, chitin and proteases involves the presence of chitinases, proteases, cellulases, β-glucanases, etc. in commercial preparations. Examples are the lysing enzymes form *Trichoderma harzianum* (provided by Sigma-Aldrich; *L1412*), which is frequently used for protoplast preparation from filamentous fungi, but also for yeast spheroplast transformation by hydrolyzing poly (1-3)-glucose of the yeast cell wall glucan (Kelly and Nurse 2011). This preparation contains β-glucanase, cellulase, protease, and chitinase activities. Lysing enzymes from *Rhizoctonia solani*, apart from β-(1-3)-glucanases, also contain protease, pectinase, and amylase activities (Liu et al. 2010). Also Driselase from *Basidiomycetes sp.* has been used for digestion of arabinoxylans by fungal glycanases (Liu et al. 2010). This commercial preparation contains laminarinase, xylanase, and cellulase (Product from Sigma-Aldrich). Bovine serum albumin (100 mg) (Sigma, St. Louis, MO, USA) and β-D-glucanase (1 g) (InterSpex Products, San Mateo, CA, USA) can be also used for cell wall digestion (Pratt and Aramayo 2002).

2.3 Getting Foreign DNA into the Fungal Cell

Filamentous fungi have gained an increased importance in recent years for production of organic chemicals, enzymes, and antibiotics. Most of strains used in industry for those purposes have been developed by screening and/or mutagenesis. Moreover, the genome of different species has been published, and genome sequences available for numerous fungi provide fast access to sequence data of individual genes to be modified. Versatility and convenience of molecular genetic tools nowadays available are constantly increasing, also as a consequence of high throughput functional genomics studies such as the *Neurospora crassa* whole genome knockout project (Colot et al. 2006). Those tools allow for transferring exogenous DNA to the genome. One of the most common techniques to transform different filamentous fungi is protoplast transformation. This procedure has a general application for different host strains.

However, alternative methods to protoplast transformation have been developed for fungal transformation, such as electroporation, biolistic transformation (Ruiz-Díez 2001), and *Agrobacterium*-mediated transformation (AMT) (Michielse et al. 2005). *A. tumefaciens* transfers a part of its DNA (transferred DNA (T-DNA)) to a high number of fungal species and its application with fungi is still increasing. AMT is an alternative for those fungi that are difficult to transform with traditional methods or for which the traditional protocols failed to yield stable DNA integration. The simplicity and efficiency of AMT allows for generation of a large number of stable transformants. Furthermore, T-DNA is integrated randomly and mainly as a single copy, which makes this method suitable for insertional mutagenesis in fungi, obtaining high homologous recombination frequencies (Michielse et al. 2005). Biolistic transformation is a powerful method when protoplasts are difficult to obtain and/or the organisms are difficult to culture. This is particularly applicable to arbuscular mycorrhizal (AM) fungi (Harrier and Millam 2001).

Nevertheless, different integration events are observed using different approaches for fungal transformation. Hence, single-copy integration events were detected when *AMT* was used for transformation (de Groot et al. 1998; Malonek and Meinhardt 2001; Meyer 2008; Meyer et al. 2003; Mullins and Kang 2001). Protoplast transformation preferentially leads to multicopy integration events (Meyer 2008; Meyer et al. 2003; Mullins and Kang 2001) and lower homologous integration than other methods (Grallert et al. 1993). The influence of the transformation technique on the outcome of the DNA integration event can determine the design of the genetic modification approach. For instance, for targeted integration or gene deletion, *AMT* would be the method of choice, since it has been shown to favor homologous recombination (HR). Nevertheless, protoplast transformation is still

the standard method in most labs (besides electroporation), because laborious vector construction and preparation largely alleviate the advantages of AMT. Moreover, protoplasting transformation would be choice if multiple copies of a gene of interest should integrate at random sites in the genome (heterologous recombination). If AMT does not result in a good efficiency of transformation, protoplast transformation using a NHEJ-deficient recipient strain could be used instead (Meyer 2008).

The first protoplast transformation was made in *Saccharomyces cerevisiae*. Hutchison and Hartwell (1967) had designed a protocol for protoplast preparation by dissolving the cell walls with a commercial glucanase preparation (Glusulase) and stabilizing the resulting protoplasts with sorbitol. The goal of this first fungal transformation was to use the protoplasts for studies on macromolecular synthesis. The next approach using protoplasts for transformation was made on a *leu2* mutant to revert the auxotrophy to prototrophy by introducing wild type DNA in the presence of calcium chloride (Hinnen et al. 1978). Later on, the use of protoplasts for transformation was extended to the filamentous members of the class *ascomycetes, N. crassa* (Case et al. 1979) and *Aspergillus nidulans* (Tilburn et al. 1983), and to several other species over the next years till nowadays. Although the original protocols have been improved, the main steps were not fundamentally changed: first, the fungal cell wall needs to be removed by enzymatic degradation. During and after this step, osmotic stabilization of the generated protoplasts is required for survival. Thereafter, several chemicals are used to render the remaining cell membrane permeable for foreign DNA. Finally, DNA is added to the protoplasts and shuttled into the cells. In the following we give an overview on protocols used for these steps, which can serve as a basis for trouble shooting of existing protocols and for development of new protocols for fungi, for which transformation has not yet been attempted or achieved. A summary of conditions used in various organisms is provided in Table 2.1.

2.3.1 Removing the Cell Wall: Protoplast Preparation

2.3.1.1 Cell Type and Growth Phase

For protoplast preparation, different cell types have been used in different species. Germinating micro- and macroconidia can be the choice for protoplast preparation (Olmedo-Monfil et al. 2004). Generally, as protoplasting involves removing the cell wall, the growth phase should be chosen in a way that the fungal cell wall is vulnerable to the attack by hydrolytic enzymes, which is more likely in an early stage after germination.

The time necessary to grow the mycelium plays an important role in protoplast preparation, and is related to the growth phase (Naseema et al. 2008). The components of the cell wall vary during different stages of the growth phase, leading to different degrees of composition/digestion of the cell wall. Moreover, components and their relative ratios in the fungal cell wall also differ between different species. Usually, the mycelium is more sensitive to lytic enzymes in the log phase (Naseema et al. 2008). In *Neurospora* sp. usually young mycelium (after 4–6 h of growth at 25–30 °C) is used to release protoplasts from hyphae after enzymatic treatment to break the cell wall (Buxton and Radford 1984; Vollmer and Yanofsky 1986). A more recent protocol uses conidial spheroplasts for transformation, growing *N. crassa* for 3 days at 34 °C followed by 2 days at room temperature (Pratt and Aramayo 2002). However, depending on the needed protoplast preparation efficiency, different alternatives can be considered. For *Aspergillus* and *Penicillium* species both germinating conidia and mycelium were used (Fincham 1989). *A. niger* was grown for 20 h (Azizi et al. 2013), while *A. nidulans* can be grown for 10 h (Mania et al. 2010), both at 30 °C. A pre-culture of *Penicillum chrysogenum* is grown for 24 h at 30 °C (Flanagan et al. 1990; Hamlyn et al. 1981; Sukumar et al. 2010). Mycelium of *Podospora anserina* (Brygoo and Debuchy 1985) and *Ascobolus immersus* (Goyon and Faugeron 1989) is used for protoplast preparation since those species do not produce conidia. Nevertheless, basidiospores (used

Table 2.1 Summary of protoplast preparation protocol for different fungal species

	Pre-growth			Lysis of cell wall				
Organism	Medium	Time	*T*	Enzymes	Time	*T*	Osmotic stabilizer	References
N. crassa	CM + 1.5 %S	4–6 h	25–30 °C	Glusulase/Novozyme 234	1 h	30 °C	Sorbitol	Case et al. (1979), Vollmer and Yanofsky (1986), Mautino et al. (1996)
A. niger	MM + Y + C + G + U	12–20 h	30 °C	Glucanex	1–2.5 h	30–37 °C	Sorbitol/NaCl/$MgSO_4$	De Bekker et al. (2009), Mania et al. (2010), Azizi et al. (2013)
A. nidulans	MM + Y + C + G + U	10 h	30 °C	Glucanex	2 h	30 °C	Sorbitol/NaCl/$MgSO_4$	Mania et al. (2010)
P. chysogenum	PDB	24 h	30 °C	Novozyme 234	2 h	RT	NaCl	Hamlyn et al. (1981), Flanagan et al. (1990), Sukumar et al. (2010)
T. harzianum	PDB	16 h	28 °C	Novozyme 234	3 h	28 °C	Sorbitol/KCl	Balasubramanian and Lalithakumari (2008), Savitha et al. (2010)
T. atroviride	PDB	15–16 h	28 °C	Lysing enzymes for *T. harzianum*	2 h	30 °C	Sorbitol	Cardoza et al. (2006)
T. reesei	M	16 h	28 °C	Lysing enzymes for *T. harzianum*	2 h	28–30 °C	Sorbitol	Gruber et al. (1990)
C. cinereus	Y + M + G	48 h	37 °C	Defined mixture cellulases + chitinases	2 h	30–37 °C	Sucrose/KCl/ Mannitol	Binninger et al. (1987)
U. maydis	Y + P + S	18 h	30 °C	Novozyme 234	[a]	30 °C	Sorbitol	Waard (1976), Eppendorf (2002)
F. graminearum	Y + P + D	12–16 h	RT	Driselase and lysing enzymes	[a]	30 °C	Sorbitol	Goswami (2012)
F. pallidoroseum	PDB	18 h	25 °C	Lysing enzymes for *T. harzianum*	3 h	30 °C	KCl	Naseema et al. (2008)
S. cerevisiae	Y + P + D	4 h	30 °C	Zymolase	20–90 min	30 °C	Sorbitol	Ezeronye and Okerentugba (2001), Tomo et al. (2013)
Ozonium sp.	Y + P + salts ($CaCl_2$ + $MgSO_4$)	36 h	28 °C	1.5 % lywallzyme + 0.5 % snailase + 1.5 % cellulose + 0.5 %lysozyme	3 h	30 °C	NaCl	Zhou et al. (2008)

CM complete medium, *MM* minimal medium, *Y* yeast extract, *C* casaminoacids, *S* sucrose, *G* glucose, *RT* room temperature, *U* uridine, *M* malt extract, *P* bactopeptone, *D* dextrose, *PDB* potato dextrose broth

[a]Protoplast formation is checked in the microscope, determining the time needed for digestion of cell wall

for *Schizophyllum commune* (Munoz-Rivas et al. 1986)), dikaryotic mycelium, or, in some cases, the very small vegetatively produced oidia can be used (Agaricales and other Basidiomycetes). *Coprinopsis cinerea (Coprinus cinereus)* was grown for 48 h at 37 °C (Binninger et al. 1987) while other Basidiomycetes as *Ustilago maydis* were grown for 18 h at 30 °C (Waard 1976) or for 24 h at 24 °C (Liu et al. 2010). For protoplasts from *Fusarium pallidoroseum* (mycelium is obtained by centrifugation for 10 min at 10,000*g* from a liquid culture), the optimal incubation time is 18 h at 25 °C (Naseema et al. 2008), while for *F. graminearum* incubation takes 12–16 h at room temperature (Goswami 2012). The optimal age of cultures reported for *T. harzianum* was 16–24 h at 25–29 °C (Balasubramanian and Lalithakumari 2008). Mycelium of *T. atroviride* was used for protoplast preparation, being 13–14 h at 28 °C the conditions for pre-growth (Cardoza et al. 2006). For *T. reesei* the optimal pre-growth is 16 h at 28 °C. *Paracoccidioides brasiliensis* was grown for 72 h (De Borba et al. 1994). For *Stagonospora nodorum*, *Cochliobolus sativus*, *Pyrenophora teres*, and *Cercospora beticola* the protocol indicates the growth of cells for 4 days at 27 °C (Liu and Friesen 2012). The choice of cell type in any fungus is a matter of experience, and the efficiency of protoplasting can hardly be predicted in advance.

However, from the examples given above, a good starting point for developing a protoplasting protocol for a new species is young mycelium from conidia germinated and grown for 12–24 h at temperatures of 25–30 °C.

2.3.1.2 Growth Conditions

In addition to the growth phase, the growth conditions applied to reach this growth phase are crucial. Besides the suitable temperature of 25–30 °C, the medium composition of the culture is important. Again, depending on the organism, different media compositions proved successful. *N. crassa* is grown in complete medium with sucrose (Case et al. 1979; Mautino et al. 1996; Vollmer and Yanofsky 1986) or Vogel's minimal medium with sucrose (Akins and Lambowitz 1985; Schweizer et al. 1981). *Acremonium chrysogenum* can be grown on a modified medium suggested by Demain and colleagues, which contains meat extract, fish meal, corn steep solids, ammonium acetate, sucrose, and glucose (Demain et al. 1963; Demain and Newkirk 1962; Hamlyn et al. 1981). *A. niger* can be grown in transformation medium (Kusters-van Someren et al. 1991) or in SAB-UU broth (sabouraud agar, pH 6.5, supplemented with uridine and uracil) (Azizi et al. 2013). Other *Aspergillus* strains were grown in minimal medium supplemented with yeast extract, casamino acids, glucose, and uridine (Hamlyn et al. 1981; Pontecorvo et al. 1953). *T. harzianum* and *T. atroviride* are grown in PDB (Potato Dextrose Broth) (Balasubramanian and Lalithakumari 2008), while *T. reesei* is grown on malt extract plates (Gruber et al. 1990). *P. chrysogenum* was grown on the medium containing mineral salts, corn steep liquor, yeast nucleic acid hydrolysates, methionine, phenylacetylethanolamine, sucrose, and agar (Macdonald et al. 1963) supplemented with yeast extract and casamino acids (Hamlyn et al. 1981) and also in PDB (Flanagan et al. 1990; Sukumar et al. 2010). A similar medium was used for *F. pallidoroseum* (Naseema et al. 2008). Nevertheless, for *F. graminearum* YPD medium (yeast extract, bactopeptone, and dextrose) is the medium of choice (Goswami 2012), as well as for *S. cerevisiae* (Ezeronye and Okerentugba 2001). Protoplasts from *C. cinerea* were prepared using oidia grown in YMG (yeast extract, malt extract, and glucose) media (Binninger et al. 1987). The medium used for *U. maydis* pre-growth is composed of yeast extract, bactopeptone, and sucrose (Eppendorf 2002; Waard 1976). PDA (Potato Dextrose Agar) was the medium used for *Lentinus lepideus* protoplast preparation (Kim et al. 2000). *Rhizoctonia solani* was grown in V8 juice agar (Liu et al. 2010), *P. brasiliensis* in PYG (peptone, yeast extract, and glucose) medium (De Borba et al. 1994), and *Pseudozyma flocculosa* in YMPD (yeast extract, malt extract, peptone, and dextrose) (Cheng and Belanger 2000) for protoplast preparation. *S. nodorum*, *C. sativus*, *P. teres*, and *C. beticola* are grown in Fries medium (Liu and Friesen 2012). Germination of spores and initial cultivation can be done on solid media (ideally

covered with cellophane to enable easy removal of mycelia) or in liquid culture.

2.3.1.3 Optimal Enzyme Combination for Lysing the Cell Wall

After reaching the optimal growth phase for protoplasting, the fungal cell wall as major obstacle for uptake of DNA has to be removed (Olmedo-Monfil et al. 2004). The structure of the cell wall is different among different fungi, requiring different enzyme combinations together with specific conditions to sufficiently degrade the cell wall and release protoplasts. Furthermore, combination of enzymes was found to be more efficient than using single enzymes for this purpose (Gallmetzer et al. 1999), which is in accordance with the presence of diverse compounds in the fungal cell wall. Therefore, the selection of enzymes is a key factor in protoplast preparation. The effectiveness of cell wall degradation depends on the combination of choice for different batches of lytic enzymes.

Traditionally, commercially available enzymes have been used for protoplast isolation from yeasts. The digestive juice from *Helix pomatia* was the first lytic enzyme preparation used, which contains muramidases and β-glucuronidases (Eddy and Williamson 1959). Later, the same enzyme mixture has been also used extensively by many other groups with *C. neoformans* and *S. cerevisiae* (Deutch and Parry 1974; Foury and Golfeau 1973; Partridge and Drew 1974; Peterson et al. 1976; Shahin 1972; Whittaker and Andrews 1969) and it was commercially available as "helicase", "sulfatase," and "glusulase" (Peberdy 1979). A second enzyme, zymolyase, commercially known as Zymolase 100T and derived from *Arthrobacter luteus*, has also been used since then for the isolation of protoplasts from different fungi, as *S. cerevisiae* (Dziengel et al. 1977; Ezeronye and Okerentugba 2001; Tomo et al. 2013) and *P. brasiliensis* (Zymolase 20T) (De Borba et al. 1994).

For *N. crassa*, Glusulase and Novozym 234, the commercial name of an enzyme mixture from *T. viride*, which was most frequently used, were the most common enzyme preparations for protoplasting (Case et al. 1979; Vollmer and Yanofsky 1986). Production of Novozym 234 (Novo Nordisk) was however discontinued and the product was replaced by "Lysing enzymes from *Trichoderma harzianum*" (Sigma-Aldrich), also known as Glucanex, in many research groups (used for *P. chrysogenum* Hamlyn et al. 1981; Flanagan et al. 1990; Sukumar et al. 2010), *P. flocculosa* (Cheng and Belanger 2000), *T. atroviride* (Cardoza et al. 2006), and *F. pallidoroseum* (Naseema et al. 2008). More recently, bovine serum albumin (Sigma) in combination with β-d-glucanase (InterSpex Products, San Mateo, CA, USA) was used for cell wall digestion of *N. crassa* (Pratt and Aramayo 2002). *A. niger* and *A. nidulans* protoplasts were prepared using Glucanex, which contains cellulase, protease, and chitinase activities (Azizi et al. 2013; Mania et al. 2010). Likewise, Glucanex was also used for protoplast preparation of *Sclerotium rolfsii* (Fariña et al. 1998). For *Trichoderma* the new mixture seems to work equally well as the Novozyme product (Steiger 2013). Both enzyme mixtures contain mainly 1,3-glucanases and chitinases, among other hydrolytic enzymes such as cellulases and proteases. For *U. maydis* cellulases and the commercial product Driselase (enzyme mixture containing cellulases, pectinase, laminarinase, xylanase, and amylase) were used for cell wall digestion (Waard 1976). Likewise, for cell wall digestion of *R. solani* (Liu et al. 2010) and *F. graminearum* (Goswami 2012), a combination of lysing enzymes and Driselase were used. For *S. nodorum*, *C. sativus*, *P. teres*, and *C. beticola* a combination of β-1,3 glucanase and Driselase (from Sigma-Aldrich) is used for cell wall digestion (Liu and Friesen 2012). Some researchers have preferred to use defined enzyme mixtures of cellulases and chitinases for preparing protoplasts of *C. cinerea* (Binninger et al. 1987). For the taxol-producing ascomycete *Ozonium,* lywallzyme was the most efficient enzyme to produce protoplasts as tested with several combinations of enzymes (Zhou et al. 2008).

The concentration of enzyme mixture necessary for digestion of cell wall has to be tested for each enzyme combination and each fungus. For *F. pallidoroseum* 20 mg of lytic enzyme/mL was reported as optimal concentration for achieving

the maximum yield of protoplasts (Naseema et al. 2008). For *T. harzianum* and *T. viride* it was shown that 5 mg/mL of the lytic enzyme Novozym 234 in osmotic stabilizer (0.6 M KCl) was the optimal concentration for protoplast preparation (Balasubramanian and Lalithakumari 2008). For *T. reesei*, 5 mg/mL of Novozym 234 were used (Gruber et al. 1990) and the same concentration of Glucanex is currently used. Hundred milligrams of bovine serum albumin (Sigma, St. Louis, MO, USA) in combination with 1 g of β-D-glucanase (InterSpex Products, San Mateo, CA, USA) were used for cell wall digestion of *N. crassa* (Pratt and Aramayo 2002).

An alternative to improve the protoplast preparation efficiency is to make a pretreatment to modify or influence the structure of cell wall. As a result, the cell wall can be more flexible or sensitive during the treatment with enzymes. Addition of 2-mercaptoethanol was found to improve the release of protoplasts (Ezeronye and Okerentugba 2001; Peberdy 1979). Likewise, pretreatment with 2-mercaptoethanol containing 0.1 M Tris and 0.1 M EDTA improved the protoplast preparation efficiency (Zhou et al. 2008). Mercaptocompounds are assumed to accelerate cell wall degradation due to the rupture of disulphide bonds of cell wall proteins (Okerentugba 1984).

Also for *S. cerevisiae* transformation, the use of lithium ions to make cell walls permeable to DNA without forming protoplasts was adopted as the alternative by some groups (Das et al. 1984; Dhawale et al. 1984; Ito et al. 1983; Limura et al. 1983).

2.3.1.4 Stabilizing Protoplasts During and After Removal of the Cell Wall

During digestion of the cell wall, fungi need to keep the osmotic balance to avoid rupture of cells. Rigid cellular walls are necessary for fungal cells to survive in hypotonic environments. When the cell wall is removed, a hypertonic environment is necessary to keep the cell stable and avoid lysis. Therefore, all solutions used for protoplast preparation have to contain an osmotic stabilizer to prevent lysis of protoplasts. At the same time, contact with any agents that could possibly damage the cell membrane once the cell wall is removed (such as traces of soap on glassware) has to be avoided. Sorbitol, at concentrations between 0.8 and 1.2 M, has been most commonly used and seems to be satisfactory for many species including *N. crassa* (Case et al. 1979; Vollmer and Yanofsky 1986; Mautino et al. 1996), *Aspergillus* sp. (Azizi et al. 2013; De Bekker et al. 2009; Mania et al. 2010), *Trichoderma* sp. (Gruber et al. 1990), *P. brasiliensis* (De Borba et al. 1994), *F. graminearum* (Goswami 2012), and *U. maydis* (Waard 1976; Eppendorf 2002). For *Aspergillus* and *Penicillium* sp., potassium chloride at concentrations between 0.6 and 0.7 M is used as standard osmotic stabilizer (Ballance and Turner 1985; Díez et al. 1987; Picard et al. 1987). A similar solution is applied for *Ozonium* sp. (Zhou et al. 2008). Magnesium sulfate at 1.2 M was also used for *A. niger, A. nidulans*, and *S. rolfsii* protoplast preparation (Fariña et al. 1998; Tilburn et al. 1983). Other alternatives are 0.5 M mannitol which was applied for protoplasting of *C. cinerea* (Binninger et al. 1987) and sucrose for *Podospora* sp. (Brygoo and Debuchy 1985), *L. lepideus* (Kim et al. 2000), *P. flocculosa* (Cheng and Belanger 2000), and *R. solani* (Liu et al. 2010) as osmotic stabilizers. For *T. virens*, sorbitol is the osmotic stabilizer of choice for protoplast preparation (Catalano et al. 2011). Nevertheless, for *T. harzianum* and *T. viride*, a test for the optimal concentration of the optimal osmotic stabilizer revealed 0.6 KCl as ideal (Balasubramanian and Lalithakumari 2008), which also works for *Trichothecium roseum* (Balasubramanian et al. 2003) and *F. pallidoroseum* (Naseema et al. 2008). Likewise, KCl as osmotic stabilizer increased the regeneration efficiency for *Talaromyces flavus* when added to regeneration medium (Santos and De Melo 1991). A similar effect was observed for *S. nodorum*, *C. sativus*, *P. teres*, and *C. beticola* (Liu and Friesen 2012). In general, it is assumed that inorganic salts are more effective for fungi while sugar and sugar alcohols are considered more advisable with yeasts and higher plants (Lalithakumari 1996). However, as the protocols reviewed above show, protoplasting is possible with both agents in

concentrations of 0.5–1 M and has to be optimized for every fungus individually. The concentration of osmotic stabilizer is critical to keep the protoplasts alive, and low concentrations of sorbitol or sucrose around 0.2 M in single layer cultures or 1 M in overlay medium can increase the fast growth of colonies, while higher concentrations could inhibit them (Ruiz-Díez 2001).

2.3.1.5 Incubation Time and Temperature for Lysing the Cell Wall

Efficient uptake of foreign DNA and subsequent integration into the genome is crucially dependent on complete removal of the fungal cell wall, but without destabilizing the vulnerable protoplasts. It is important to determine the temperature range in which a maximal rate of lysing cell walls is achieved with different enzymes. The enzymolysis temperature for most filamentous fungi was found to be between 24 and 35 °C (Sun et al. 2001). In addition to osmotic shock, protoplasts can also be damaged by temperature shocks, which have to be avoided. The incubation time necessary to break the cell wall varies from 2 to 3 h in most protocols for fungi, with increasing protoplast production of mycelia gradually until 3 h. Longer exposure time (also in dependence of enzyme combination) was reported to lead to rupture of protoplasts due to damage of the cell membrane (Zhou et al. 2008). In contrast, shorter exposure times to lytic enzymes resulted in higher capacity to regenerate protoplasts (Zhou et al. 2008). However, at the same time efficiency of DNA uptake and genomic integration is likely to decrease if the cell wall is not completely removed. Therefore the optimum time and temperature for a given species, cell type, and enzyme mixture used has to be found.

For *Ozonium* sp., 3 h at 30 °C were found to be the optimal conditions for maximum protoplast release (Zhou et al. 2008), as well as for *S. nodorum*, *C. sativus*, *P. teres*, and *C. beticola* (Liu and Friesen 2012). For *F. pallidoroseum*, 3 h at 30 °C with constant stirring at 30 rpm were the conditions used for protoplast preparation, while after 4 h the mycelium was completely lysed (Naseema et al. 2008). Here it is important to note that also shearing forces due to stirring, but also subsequently due to rapid pipetting can cause the newly formed protoplasts to rupture. The maximum release of protoplasts from *T. harzianum* and *T. viride* was reported after 3 h of incubation at 100 rpm, 28 °C and pH 5.5 (Balasubramanian and Lalithakumari 2008). *T. reesei* and *T. virens* protoplast preparation requires an incubation time of 2 h at 30 °C with only gentle agitation, as well as for *A. niger* and *A. nidulans* protoplasts (Azizi et al. 2013; Mania et al. 2010) and *C. cinerea* (Binninger et al. 1987). However, for *N. crassa*, 60–90 min at 30 °C was enough for protoplast release (Pratt and Aramayo 2002; Vollmer and Yanofsky 1986). For *P. chrysogenum*, 2 h at room temperature are sufficient for removal of the cell wall (Hamlyn et al. 1981; Flanagan et al. 1990; Sukumar et al. 2010). Digestion of the *L. lepideus* cell wall requires an incubation time of 6 h of the mycelium for the release of protoplasts (Kim et al. 2000). The time and temperature used for *R. solani* protoplast release consist on a first incubation at 37 °C for 15 min followed by 34 °C during 105 min incubation (Liu et al. 2010). In case of *S. rolfsii* cell wall digestion requires 1 h of incubation at 45 °C (Fariña et al. 1998). For *U. maydis* the exact time for digestion of cell wall is determined by checking the number of protoplast released under the microscope, using around 30 °C for incubation (Waard 1976; Eppendorf 2002), likewise for *F. graminearum* (Goswami 2012).

2.3.1.6 Evaluation and Storage of Protoplasts

In order to achieve high transformation efficiency, protoplasts should be checked under the microscope in order to confirm their integrity. Usually this is done by adding distilled water to the microscope slide. Due to the altered osmotic pressure, protoplasts will lyse, while cells with insufficiently degraded cell wall will remain intact and prevent efficient uptake of DNA, which will decrease overall transformation efficiency. If this is the case, incubation time should be increased (Becker and Lundblad 2001).

Concentration of protoplasts can be checked directly under the microscope or in a hemocytometer. The ratio of protoplasts to DNA can strongly influence transformation efficiency. While a high number of protoplasts decreases the amount of DNA per cell and hence likelihood of integration, a low number of protoplasts and/or high DNA concentration will increase the total number of transformants obtained although their number per µg of DNA is lower (Forsburg 2003). However, increased amounts of DNA per protoplast can also result in multicopy integration.

After incubation for digestion, protoplasts are normally purified by filtration through glass wool, glass filters, or nylon filters such as Miracloth (Calbiochem), followed by gentle centrifugation to remove the supernatant and resuspension of the protoplasts in a solution containing osmotic stabilizers. The speed and time of centrifugation depend on how sensitive protoplasts are. Longer centrifugation times normally result in a higher number of protoplasts to be obtained, but at the risk of damaging them. For *T. reesei* 10 min at 600*g* is appropriate, while for *F. pallidoroseum* 6 min at only 100*g* is used (Naseema et al. 2008). In general, it is important to avoid shearing forces by using swing out rotors instead of fixed angle rotors, as the sliding along the wall of the tube might kill protoplasts. Similarly, vigorous vortexing or pipetting has to be avoided.

Once protoplasts are stabilized with sorbitol, they can be snap frozen and stored at −70 to −80 °C for many fungi. With *S. cerevisiae* and *S. pombe* this is commonly done with tolerable loss in transformation efficiency (Altherr et al. 1983; Gietz and Schiestl 2007; Jimenez 1991).

Neurospora protoplasts have been found to remain viable indefinitely at −70 °C, and so a single batch can be used for several successive transformation experiments (Pratt and Aramayo 2002; Vollmer and Yanofsky 1986). Protoplasts of *Aspergillus* spp. are reported to be similarly robust (De Bekker et al. 2009). However, this is not the case for all protoplast preparations. For example, for transformation of *T. reesei* freshly prepared protoplasts are required, because storage of protoplasts reduces drastically their efficiency (Penttilä et al. 1987).

2.3.1.7 Uptake of DNA/Transformation

The common protocol for transformation of protoplasts starts with mixing the purified DNA (either linear or circular double-stranded), with the protoplast suspension and a solution containing polyethylene glycol (PEG). In contrast to transformation protocols for yeast, using carrier DNA is not common during transformation of filamentous fungi. The incubation time necessary is between 15 and 30 min on ice for most of fungi in order to allow for DNA attachment to protoplasts (Ruiz-Díez 2001). Although double-stranded DNA is normally used, single-stranded DNA was successfully transformed into *Saccharomyces* sp. (Singh et al. 1982) and *A. immersus* (Goyon and Faugeron 1989).

The stabilization buffer, in which the protoplasts are suspended, is usually composed of calcium ions and an osmotic stabilizer. Calcium ions are assumed to be responsible for opening channels or pores in the cell membrane to facilitate DNA uptake (Olmedo-Monfil et al. 2004). The osmotic stabilizer is present to keep the osmotic balance, which is usually maintained by the cell wall (see above). PEG is considered responsible for clumping and fusion of protoplasts, precipitation of DNA and consequent induction of interaction between DNA and the cell surface (Bird 1996; Fincham 1989). Although a more efficient alternative to deliver DNA to the cells was proposed, the role of PEG in DNA uptake remains largely unknown (Radford et al. 1981). Nevertheless, PEG is important for efficient transformation and if poor frequencies are obtained, different lots of PEG should be tested (Becker and Lundblad 2001). Generally, lower molecular weight PEG (such as 3,350) works better than high molecular weight PEG (such as 8,000), but also here optimization for an individual species may improve results. For yeast transformations, it is recommended to remove PEG prior to plating the transformation mixture in order to increase efficiency (Becker and Lundblad 1997). This may also be beneficial for transformation of filamentous fungi.

In an alternative protocol, DNA can be encapsulated in liposomes (artificially constructed lipid vesicles), which can fuse with protoplasts.

Nonetheless, this is a time-consuming procedure compared to the routine protocols using free DNA (Fincham 1989).

Additional components can be added to the transformation mixture, such as 1 % (w/v) dimethyl sulfoxide (DMSO), to stimulate electrofusion of protoplasts (Case et al. 1979; Nea and Bates 1987), or 0.05–0.1 mg of heparin per mL (Kinsey and Rambosek 1984) in successful protocols only for *Neurospora*. Although several functions of heparin are known, such as assisting serine protease inhibitors, it is unknown how this can influence transformation efficiency (Bird 1996). Nevertheless, there is no evidence to confirm that these additional components could improve the yields of transformants in other fungi.

The DNA concentration needed for transformation usually varies between 3 and 10 μg, with 10 μg being the ideal concentration, for example, for *T. reesei*, although lower amounts also may yield satisfactory results. Regarding protoplast concentration, variations according to laboratory preferences are common. Usual amounts range from 5×10^7–5×10^8 (Kubicek and Harman 1998) to 1×10^8–1×10^9 mL^{-1} (Gruber et al. 1990).

Also the final concentration of PEG is critical for the efficiency of DNA uptake. Therefore, PEG added should be adjusted to final volume of the solution including protoplasts and DNA (Gietz and Schiestl 2007). The concentration of calcium chloride can vary from 10 mM, as most commonly used for *S. cerevisiae*, *Aspergillus* sp., and other filamentous fungi, to 50 mM for *Neurospora* species (Fincham 1989). PEG solution can contain a final concentration of 25 % (w/v) PEG 6000 (Gruber et al. 1990), although up to 10 volumes of 40 % (w/v) PEG 4000 can be used for majority of fungal species (Fincham 1989). Nevertheless, a protocol for *S. commune* specifies only a little over 1 volume of 44 % (w/v) PEG 4000 (Munoz-Rivas et al. 1986). pH seems to play a role to keep protoplasts stable and a range of 6–8 is used for different species of fungi (Ruiz-Díez 2001). Some protocols include the addition of lipofectin, a liposome preparation formulated from cationic lipids (Bethesda Research Laboratories, Gaithersburg, MD), increasing the transformation efficiency, as reported for *N. crassa* (Selitrennikof and Sachs 1991).

After 15–30 min of incubation with exogenous DNA, protoplasts, and PEG, some protocols include the addition of 1–2 mL of PEG, followed by an incubation time of 5–30 min (depending on the species) at room temperature (Gruber et al. 1990). However, normally protoplasts are quite stable and rounds for washing can be increased for convenience. Finally, stabilization buffer is added to the solution and in case of using an agar-overlay, mixed with the overlay medium or alternatively spread out on selective medium (Gruber et al. 1990). Interestingly, also the brand of agar used for plate preparation can influence transformation efficiency (likely due to impurities) and should be considered when optimizing protocols.

2.3.1.8 Incubation Time and Medium for Protoplast Regeneration

Regeneration of protoplasts can be improved depending on the media prior to application to selective medium. Therefore, protoplasts are usually transferred to regeneration media without selective pressure. However, in many cases, transformed protoplasts are transferred directly to media complementing an auxotrophic marker or containing a drug depending on the selectable marker gene used on the introduced DNA construct, without prior cultivation on media lacking the selection reagent. In these cases, recovery of the fungus from the protoplast state is often called regeneration. The media used for regeneration have to be osmotically buffered to allow the recovery of protoplasts.

N. crassa transformed protoplasts can be regenerated overnight at 25 °C in regeneration solution (Vogel's salts + Sucrose + $MgSO_4$ + any necessary metabolic supplements) (Pratt and Aramayo 2002). Regeneration of *A. niger* protoplasts is done on a nonselective medium (minimal medium pH 6.0, sucrose and agar) (De Bekker et al. 2009). *T. reesei* protoplasts after transformation were recovered on malt extract medium containing sorbitol, hygromycin B, and uridine (Guangtao et al. 2009). Later on, once protoplasts are regenerated, only those ones which harbor the transforming DNA can grow in the medium including a selection reagent. The maintenance of osmotic balance is critical for recovering protoplasts. Additionally, for protoplast

recovery in *Ozonium* sp., 0.1 % (w/v) Triton X-100 and 0.04‰ (w/v) sodium deoxycholate were used as colony restrictors. The use of these detergents or others is important to avoid spore clumping, in order to isolate single transformant colonies (Jensen et al. 2013). However, such colony restrictors are most often only added for single spore isolation steps after initial transformant isolation. Thereby decreased recovery of protoplasts due to a destabilizing effects of, for example, the detergent Triton X-100 can be avoided.

2.4 Concluding Remarks

Protoplasting is well established as a method for fungal transformation nowadays. Therefore, to develop a transformation system for a fungus, protoplasting is a good choice. Nevertheless, for many species there is considerable room for improvement with efficiency of protoplast transformation and we provided some strategies to optimize existing protocols. Developing and optimizing protoplast transformation with fungi still remains of process of trial and error to some extent and due to the varying cell wall composition and maybe also some defense mechanisms, protocols cannot be generalized.

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Trichoderma Transformation Methods

3

Mónica G. Malmierca, Rosa E. Cardoza, and Santiago Gutiérrez

3.1 General Methods

Most of the transformation techniques currently available for bacteria, e.g., electroporation (Schuster et al. 2012), biolistic transformation (Lorito et al. 1993; Te'o et al. 2002), and transformation by the use of shock waves (Magaña-Ortíz et al. 2013), also have been reported for *Trichoderma*. However, currently the most widely used and optimized procedures for *Trichoderma* strains are based on protoplasts and the *Agrobacterium*-mediated transformation (Cardoza et al. 2006).

Thus, in the methods described below, transformations of *Trichoderma* strains by protoplasts and mediated by *Agrobacterium* are explained in detail. Eventually, both procedures could be used to transform a particular strain. However, usually one strain can be transformed more efficiently using one of these two methods. The explanation for this phenomenon is still unknown, but the structure and composition of the cell wall as well as some properties like growth rate in a particular culture medium or the nutrient requirements would be important points to take in consideration in order to transform a particular strain.

Also, other alternative techniques are briefly described.

3.2 Detailed Procedure Description

3.2.1 Transformation of *Trichoderma* Mediated by Protoplasts

These procedures based on the isolation of *Trichoderma* protoplasts have been developed by the improvement of several methods previously described for *Trichoderma reesei* (Penttila et al. 1987; Gruber et al. 1990) or *Trichoderma* spp. (Sivan et al. 1992; Cardoza et al. 2006).

One of the limiting steps in this transformation technique is obtaining the protoplasts. For this reason, both the growth conditions and composition of the culture media have to be optimized for each strain. Other factors, as incubation temperature, time of growth, viscosity of the selection media, concentration of the lytic enzymes, and composition of solutions for release and purification of protoplasts, are critical factors that have to be observed.

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3.2.1.1 Growth Conditions and Protoplasts Formation

1. Inoculate plates of a complex media, usually PDA or PPG media, with 10^6–10^7 conidia per plate. Incubate at 28–30 °C during 3–6 days, depending on the strain (Table 3.1).
2. Collect conidia from one plate and inoculate in 100 mL of CM medium (approximately 10^6–10^7 conidia/mL) in a rotary shaker at 250 rpm and 28 °C during 13–16 h (Table 3.1). Be sure that most of the conidia have germinated and avoid the formation of closed pellets. In case of pellet formation, time and/or temperature of incubation, composition of the medium or speed of the shaker have to be optimized. Under these standardized conditions, between 1 and 2.5 g of mycelia will be recovered from 100 mL of culture.

   For *T. reesei* and *T. parareesei*, conidia will be spread on MA plates (about 5×10^6 conidia per plate) over a sterile cellophane membrane covering the plate surface. Incubate for 16–22 h (depending of the strain) at 30 °C. About five plates will be needed to get enough amount of protoplasts.
3. Filter the mycelia through a sterile nylon filter (25–30 μm of pore diameter) and wash once with 0.9 % NaCl and once more with TLT (Washing buffer: 10 mM sodium phosphate buffer, pH 5.8; 0.6 M $MgSO_4$). Then, resuspend 0.5 g of mycelia in 50 mL of TPT (protoplasts buffer: 10 mM sodium phosphate buffer, pH 5.8; 0.8 M $MgSO_4$) with or without the addition of dithiothreitol (DTT) (see Note 1). Incubate the suspended mycelia at 30 °C in a rotary shaker at 250 rpm for 2 h (Table 3.1).
4. Collect the mycelia by centrifugation at 7,500 × *g* for 5 min.
5. Wash the DTT-treated mycelia with TPT to remove the DTT and recover the mycelia as in step 4.
6. Resuspend the mycelium in 20 mL of TPT containing lytic enzymes (Lysing enzymes, catalog # L-142, Sigma, USA) at concentrations between 5 and 15 mg/mL (Table 3.1). Incubate the mixture (mycelium + lytic enzymes) at 30 °C for 0.5–2 h at low speed (80–100 rpm), to allow for release of the protoplasts. Previously to this step, the optimal magnesium sulphate concentration in TPT and TLT has to be determined to get the highest number of protoplast. In the case of *T. arundinaceum*, 0.7 M NaCl was used instead of magnesium sulphate (Table 3.1).
7. Check the protoplast formation at each hour using a light microscope (see Note 2). Once the protoplasts have been released, collect them by filtration (through filters with 25–30 μm pore diameter) and dilute 1:5 with ST buffer (10 mM Tris–HCl, pH 7.5; 1 M sorbitol) (see Note 3). Pellet the protoplasts by 10 min centrifugation at 3,000 × g.
8. Wash the protoplasts twice with ST and then once with STC (ST containing 20 mM $CaCl_2$). Proceed to pellet the protoplasts by centrifugation as in step 7.
9. Resuspend the protoplasts in STC at a concentration from 5×10^7 to 1×10^8 protoplasts/mL. Add 1/10 of volume of PTC (10 mM Tris–HCl, pH 7.5; 20 mM $CaCl_2$; 60 % polyethylene glycol 6000).

3.2.1.2 Protoplasts Transformation

1. Mix 100 μL of the protoplast suspension with 10 μg of plasmid (see Note 4).
2. Maintain the mixture on ice for 20 min and add 500 μL of PTC. Mix gently and incubate at room temperature for 20 min.
3. Dilute the mixture with 600 μL of STC and then mix aliquots of the final reaction with 5 mL of the appropriate regeneration medium (see Note 5). Spread as overlays on plates containing 5 mL of the same medium.
4. Maintain the plates at room temperature during 5–10 min to solidify the medium. Incubate at 28–30 °C during 4–6 days, to allow for regeneration of protoplasts and growth of the colonies (see Notes 6–8; Table 3.2).
5. Check the mitotic stability of the transformants: transfer them to a new Petri dish containing twice the concentration of the antibiotic used to select them. Allow them to grow and transfer the colonies to a fresh medium without antibiotic and, finally, to a fresh medium plus antibiotic. At this point those transformants are considered mitotically stable.

Table 3.1 Optimal conditions used to obtain protoplasts in different *Trichoderma* strains

Strain	Growth in solid medium (28 °C)	Growth in CM (spores/mL, h)[a]	$MgSO_4$ in TPT	DTT treatment	Lytic enzyme concn (mg/mL)[c]	Protoplast yield (protoplasts/mL)	Transformants/μg DNA[b]
T. harzianum T34	PPG, 3 d	10^7, 17	0.8 M	25 mM 2 h/30 °C	5 (2 h)	$1–2.5\times10^8$	60/30
T. atroviride B11	PDA, 3 d	5×10^6, 15	1.0 M	–	12 (3–4 h)	$1–5\times10^6$	40/15
T. longibrachiatum T52	PDA, 5–6 d	10^7, 17	0.8 M	50 mM 2 h/30 °C	7.5 (2 h)	$1.2–1.6\times10^8$	70/30
T. asperellum T53	PDA, 4–5 d	5×10^6, 15	1.0 M	–	10 (3–4 h)	$5\times10^6–1\times10^7$	45/10
T. parareesei T6	PPG, 4–5 d	5×10^6, 16–22*	1.2 M	–	10 (1–2 h)	$1–5\times10^7$	nd/5
T. reesei	PPG, 4–5 d	5×10^6, 20–22*	1.2 M	–	10 (0.5–1 h)	$1–2.5\times10^8$	600–800
T. arundinaceum IBT 40837	PPG, 5–7 d	10^9, 24	NaCl 0.7 M	–	5 (14 h) 25.0 driselase 0.05 chitinase	2×10^7	20/nd
T. brevicompactum IBT 40841	PPG, 5–7 d	10^7, 24	1 M	–	12 (3 h)	$<10^4$	0/nd

[a]Growth in CM medium was performed at 28 °C and 250 rpm. *Indicates growth in the surface of Petri dishes containing MA medium covered with a cellophane membrane
[b]These data refer to the efficiency observed when using hygromycin B/phleomycin. For *T. reesei*, the efficiency range was obtained when the *pyrG* marker was used in the transformation
[c]Lysing enzymes from Sigma catalog # L-142 (Sigma, USA) were used in all the strains used except for *T. arundinacerum*

Table 3.2 Procedures and markers used to transform different *Trichoderma* strains

Strain	Transformation procedure	Marker gene	Phenotype of transformants	References
T. reesei	AMT	*hph*	Hygromycin B^R	de Groot et al. (1998)
				Zhong et al. (2007)
T. atroviride	"	*hph*	"	de Groot et al. (1998)
				Cardoza et al. (2006)
T. harzianum	"	*hph*	"	Cardoza et al. (2006)
				Yang et al. (2011)
T. longibrachiatum	"	*hph*	"	Cardoza et al. (2006)
T. asperellum	"	*hph*	"	Cardoza et al. (2006)
T. brevicompactum	"	*hph*	"	Tijerino et al. (2011a)
T. arundinaceum	"	*ble*	$Phleomycin^R$	Malmierca et al. (2013)
T. longibrachiatum	"	*ble*	"	Cardoza et al. (2006)
T. asperellum	"	*ble*	"	Cardoza et al. (2006)
T. reesei	Protoplast	*hph*	Hygromycin B^R	Penttila et al. (1987)
T. viride	"	*hph*	"	Zhu et al. (2009)
T. harzianum	"	*hph*	"	Cardoza et al. (2006)
T. longibrachiatum	"	*hph*	"	Cardoza et al. (2006)
T. arundinaceum	"	*hph*	"	Malmierca et al. (2012, 2013)
T. harzianum	"	*ble*	$Phleomycin^R$	Cardoza et al. (2006, 2007)
T. atroviride	"	*ble*	"	Cardoza et al. (2006)
T. longibrachiatum	"	*ble*	"	Cardoza et al. (2006)
T. asperellum	"	*ble*	"	Cardoza et al. (2006)
T. parareesei	"	*ble*	"	Gutiérrez S (unpublished data)
T. harzianum	"	*amdS*	Growth in acetamide	Cardoza R.E. (unpublished data)
T. atroviride	"	*nptII*	$Geneticin^R$	Gruber et al. (2012)
T. reesei	"	*pyrG*	Uridine prototrophy	Gruber et al. (1990)
T. reesei	"	*Hxk1*	Growth on mannitol	Guangtao et al. (2010)
T. longibrachiatum	Electroporation	*hph*	Hygromycin B^R	Sánchez-Torres et al. (1994)
T. harzianum	Biolistic	*hph*	"	Lorito et al. (1993)
T. reesei	Shock wave	*hph*	Hygromycin B^R	Magaña-Ortíz et al. (2013)

AMT. Agrobacterium mediated transformation

3.2.2 Transformation of *Trichoderma* Mediated by *A. tumefaciens*

1. Electroporate *A. tumefaciens* AGL1 with constructs containing the T-DNA region [e.g., plasmids pUR5750 (de Groot et al. 1998) and pUPRS0 (Cardoza et al. 2006)] according to Mozo and Hooykaas (1991).
2. Grow the transgenic *Agrobacterium* strains overnight at 30 °C on LB plates supplemented with 50 µg/mL kanamycin, 100 µg/mL carbenicillin, or 25 µg/mL rifampicin.
3. Streak out the cells from a single colony on a minimal medium plate containing the appropriate antibiotics. *Agrobacterium* minimal medium (MM) contains per liter: 10 mL potassium–buffer, pH 7.0 (200 g/L K_2HPO_4, 145 g/L KH_2PO_4), 20 mL magnesium–sodium solution (30 g/L $MgSO_4 \cdot 7H_2O$, 15 g/L NaCl), 1 mL 1 % $CaCl_2 \cdot 2H_2O$ (w/v), 10 mL 20 % glucose (w/v), 10 mL 0.01 % $FeSO_4$ (w/v), 5 mL trace elements (100 mg/L $ZnSO_4 \cdot 7H_2O$, 100 mg/L $CuSO_4 \cdot 5H_2O$, 100 mg/L H_3BO_3, 100 mg/L $MnSO_4 \cdot H_2O$, 100 mg/L $Na_2MoO_4 \cdot 2H_2O$), 2.5 mL 20 % NH_4NO_3 (w/v), and 15 g/L bacto-agar (Difco, USA) at pH 7.5 (Hooykas et al. 1979).
4. Incubate the plates at 30 °C for 1–2 days. Inoculate several colonies from these plates in

liquid minimal medium containing 50 μg/mL kanamycin and incubate at 30 °C and 250 rpm for 24 h. Collect bacteria by centrifugation and resuspend in induction medium (IM = MM + 10 mM glucose) containing 40 mM MES pH 5.3, 0.5 % glycerol (w/v), and 200 μM acetosyringone (AS) (Mozo and Hooykaas 1991) to an optical density at 660 nm of 0.5 absorbance units. Then, incubate this bacterial suspension for 6 h at 30 °C in a rotary shaker (250 rpm) to pre-induce the virulence of *A. tumefaciens*.

5. Dilute conidia from *Trichoderma* in double distilled water to a final concentration of 10^7 conidia/mL. Then, mix 50 μL of this suspension with 50 μL of the *Agrobacterium* cell suspension from step 4. To confirm if transformation of fungal conidia by *Agrobacterium* is dependent on T-DNA transfer, a negative control has to be included in which AS, the virulence inducer, has been omitted.
6. Subsequently, spread the mixtures onto nitrocellulose filters (47 mm diameter nitrocellulose black filters, 0.8 μm pore diameter) (Millipore, Germany) placed on IM plates (1.5 % bacto-agar) containing 5 mM glucose and 200 μM AS. Incubate the plates at 18–20 °C for at least 40 h. Then, transfer the filters to TSA plates (1.5 % bacto-agar) containing 300 μg/mL cefotaxime to inhibit *Agrobacterium* growth, and the appropriate antibiotic to select the *Trichoderma* transformants (Table 3.2).
7. Incubate at 28 °C during 5–6 days.
8. Check the mitotic stability of the transformants as described for the protoplast mediated transformation.

3.3 Alternative *Trichoderma* Transformation Procedures

3.3.1 Protoplast Electroporation (Sánchez-Torres et al. 1994)

1. Protoplasts were isolated as indicated in Sect. 3.2.1.1. Thus, once the protoplast were released (step 6, Sect. 3.2.1.1), they were pellet by centrifugation at 3,000 × *g* for 10 min and then resuspended in SP solution (1 M sorbitol, 1 % (w/v) PEG 8000) to give a concentration of 1×10^8 protoplast/mL. Mix the protoplasts with the transforming DNA and carrier DNA (salmon sperm DNA). Apply an electric pulse through a Gene Pulse device (Bio-Rad Laboratories, USA) at 25 mF, 800 Ω, and 2.8 kV/cm as electrical parameters.
2. Dilute protoplasts using STC and plate them in the appropriate selective medium and incubate at 28–30 °C during 4–6 days.

3.3.2 Biolistic (Lorito et al. 1993)

1. Sporulate the *Trichoderma* strain by incubation on PDA medium at 28 °C for 7–14 days.
2. Dispose seven portions containing 5×10^7 to 1×10^8 conidia on PDA plates to align with the seven barrels of the Hepta Adaptor (Bio-Rad, USA) and leave to dry. Prepare a mixture of DNA (from 100 to 1,000 ng) precipitated with tungsten particles (0.7 μm mean diameter).
3. Resuspend the DNA mixture in 100 % ethanol, and use an aliquot for bombardment with the Bio-Rad Hepta Adaptor system with seven barrels for particle launch.
4. Incubate the plates for 5–6 h before overlaying with PDA containing the appropriate concentration of antibiotic for selection and incubate 3–5 days more.

3.3.3 Shock Waves (Magaña-Ortíz et al. 2013)

1. Mix 5×10^3 to 5×10^4 conidia and transforming DNA (50 μg/mL) and expose to 50 shock waves, generated by a Piezolith 2300 shock generator (Richard Wolf GmbH, Germany), consisting of a positive pressure peak of 150 MPa with a phase duration of 0.5–3 μs, followed by a decompression pulse of up to 20 MPa and a phase duration of 2–20 μs.
2. Dilute conidia and inoculate on 3 M cellulose filters placed on plates containing minimal medium without selective pressure and incubate for 24 h at room temperature. Transfer the filters to fresh medium with the appropriate antibiotic to select the transformants and incubate at 28–30 °C.

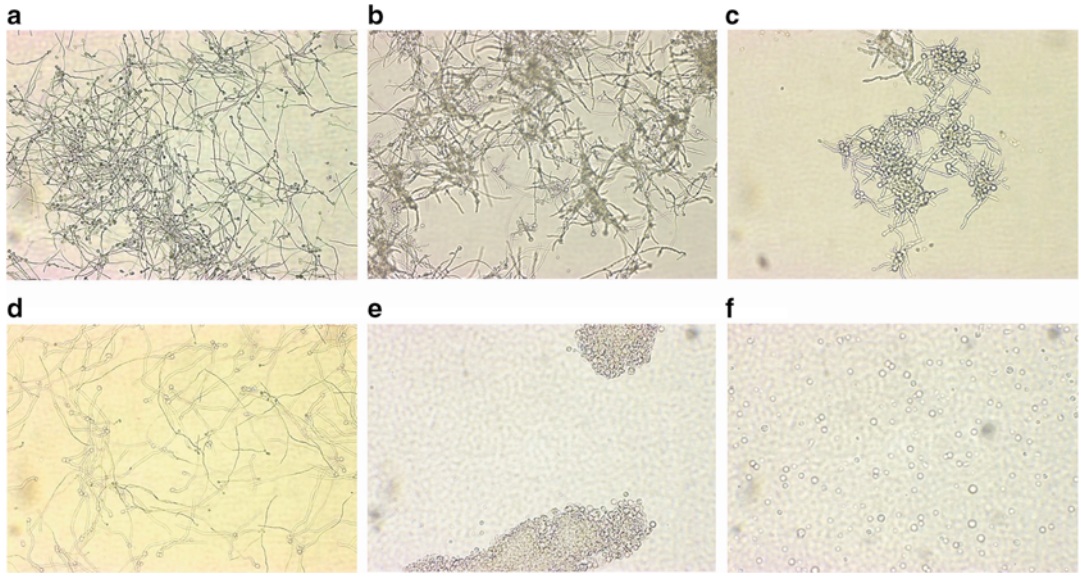

Fig. 3.1 Growth of several *Trichoderma* strains in liquid CM medium. (**a**) *T. harzianum* T34; (**b**) *T. atroviride* T11; (**c**) *T. asperellum* T53; (**d**) mycelia from *T. harzianum* T34 strain treated with 25 mM DTT; (**e**) protoplast formation from *T. harzianum* T34 strain with 1.2 M $MgSO_4$; (**f**) protoplast formation of *T. harzianum* T34 strain with 0.8 M $MgSO_4$

3.4 Notes

Note 1. DTT is used to break disulphide bonds, and is indicated to reduce the time of incubation and the concentration of lytic enzymes employed to get protoplasts. This step is only needed for some *Trichoderma* strains, e.g., *T. harzianum* and *T. longibrachiatum*.

Note 2. The microscopic observation of the mycelium during incubation with the lytic enzymes is the key starting point in the development of fungal transformation procedures (Fig. 3.1).

Note 3. Dilution of protoplasts at this step will help to recover a higher percentage of protoplasts after the first centrifugation step.

Note 4. Linearized plasmids can increase the transformation efficiency, by the generation of DNA ends which show a higher recombinogenic potential than the undigested circular plasmids. Moreover, for some strains, stability of the transformants is higher than that obtained when using undigested plasmids (Cardoza et al. 2006).

Usually, plasmids expressing dominant markers (i.e., antibiotic resistance genes) include strong fungal promoters to allow a suitable expression of the marker genes. These promoters have been isolated from fungi based in their high level of expression, and normally they also drive a high expression level in other fungal species. Examples of some of these promoters are the *gpd* gene promoter from *Aspergillus nidulans* (Punt et al. 1987), the *pki* gene promoter from *T. reesei* (Mach et al. 1994), the *gdh* gene promoter from *Aspergillus awamori* (Cardoza et al. 1998), and the promoter of *tss1* gene from *T. harzianum* (Cardoza et al. 2007).

Note 5. For the selection of transformants when using a dominant marker, complex media are normally chosen (e.g., TSA + Sorbitol, TSA + Sucrose, MA + Sorbitol) containing the appropriate antibiotic (the concentration of the antibiotic has to be optimized for each strain). When an auxotrophic marker is used, strains should be grown on *Trichoderma* minimal medium (Penttila et al. 1987) to select the recombinant strains by complementation of the auxotrophy.

Note 6. When resistance to phleomycin is used as a selection marker, an incubation of the transformation plates at 4 °C for as long as 12 h will increase the selectivity of the antibiotic.

Note 7. When the selection of transformants is based on resistance to an antibiotic, the use of media containing salts as osmotic stabilizers (NaCl, KCl, etc.) can result in a strong increase in background resistance of the WT to the antibiotic. Thus, in these cases, media containing sucrose or sorbitol as osmotic stabilizers are recommended.

Note 8. Another important point to consider is the viscosity of the medium used to select the transformants. A balance has to be established between the agar concentration of the selective medium and the PEG concentration used in the transformation mixture. High concentrations of both agar and PEG will reduce the efficiency of transformation. In addition, other parameters as the water quality and the purity of the culture media and agar have to be considered.

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Transformation of *Mucor circinelloides f. lusitanicus* Protoplasts

4

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4.1 Introduction

The current success of *Mucor circinelloides f. lusitanicus* as a fungal model system would have not been possible without the early experiments in which a leucine auxotrophic mutant (R7B) (Roncero 1984), was transformed with a genomic library constructed in a yeast-based vector (van Heeswijck and Roncero 1984). The corresponding gene was isolated and characterized (Roncero et al. 1989), and this settled the basis for future developments. The original transformation system was based on the PEG-mediated method of DNA transfer to protoplasts, which was the most popular one used in other fungal species at that time. *M. circinelloides* became then the first Mucoral fungus to be efficiently transformed. Later on, a great effort was made to find new *M. circinelloides* mutants derived from R7B, and the isolation of new selectable gene markers (*see* Table 4.1). The result was a comprehensive number of potential recipient strains and molecular markers with which the analysis of different biological problems could be achieved. These include different aspects of basic and applied biology such as carotenoid biosynthesis and regulation (Iturriaga et al. 2001; Csernetics et al. 2011), blue-light regulation (Silva et al. 2006, 2008), lipid accumulation and metabolism (Xia et al. 2011; Rodríguez-Frómeta et al. 2013), dimorphism and differentiation (Wolff et al. 2002; Ocampo et al. 2009, 2012), or siRNA silencing (Nicolás et al. 2009; Calo et al. 2012), among others.

The next step was trying to increase the transformation efficiency and reliability. This has been done during the last 30 years in two ways: by modifying the original procedure or by adapting new transformation techniques that were progressively being developed (Gutiérrez et al. 2011). *M. circinelloides* has been shown to be a natural competent organism in contrast with other related species or genera. A good example is that today, germinated and ungerminated spores of this fungus can readily be transformed by biolistic (González-Herrnández et al. 1997) or *Agrobacterium*-mediated (Nyilasi et al. 2005) methods, although at a lower efficiency than protoplast-mediated ones.

A distinctive feature of the *M. circinelloides* transformation system (and other Mucoromycotina)

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Table 4.1 Strains and plasmids discussed in this chapter

Strain	Genotype	Origin	Phenotype	Transforming DNAs derived from plasmids	Genetic marker	References
CBS 277.49	Wild type	A.F. Blakeslee's collection	Yellow			Harris (1948), Schipper (1976)
R7B	*leuA*$^-$	CBS277.49 (UV)	Yellow	pLeu4	LeuA	Roncero (1984)
R5A	*met*$^-$	CBS277.49 (UV)	Yellow	pMcM20	Met	Anaya and Roncero (1991)
MS8	*leuA*$^-$, *carRP*$^-$	R7B (ICR170)	White	pLeu4	LeuA	Rodríguez-Sáiz et al. (2004)
MS12	*leuA*$^-$, *pyrG*$^-$	R7B (NG)	Yellow	pLeu4, pEPM1, pEPM9	LeuA, PyrG	Benito et al. (1992, 1995)
MS23	*leuA*$^-$, *pyrG*$^-$, *carB*$^-$	MS12 (NG)	White	pLeu4, pEPM1, pEPM9	LeuA, PyrG	Rodríguez-Sáiz et al. (2004)
MS41	*carB*$^-$, *pyrFa*$^-$	CBS277.49 (NG)	White	pAVB20	PyrF	Velayos et al. (1998)
MS46	*carB*$^-$, *pyrFb*$^-$	CBS277.49 (NG)	White	pAVB20	PyrF	Velayos et al. (1998)
MU402	*leuA*$^-$, *pyrG*$^-$	R7B (NG)	Yellow	pLeu4, pEPM1	LeuA, PyrG	Nicolás et al. (2007)
MU520	*pyrG*$^-$	MU402 (targeted integration of LeuA gene)	Yellow	pLeu4	PyrG	V. Garre (unpublished data)

is that plasmids tend to be self-replicative (van Heeswijck 1986; Papp et al. 2010), in contrast with what it happens in other fungal transformation systems in which the fate of the transforming DNA is always the integration in the genome. This could be an advantage or a handicap depending on the kind of experiments to be done. Although REMI (restriction enzyme-mediated integration) (Papp et al. 2013), or *Agrobacterium*-mediated (Nyilasi et al. 2005) methods have been used to force integration of DNA fragments into the genome of *M. circinelloides*, the use of linear DNA fragments works equally well with the PEG-mediated or electroporation transformation methods. These linear DNA fragments are used either to disrupt genes or to target the insertion of selected genes into a particular locus by homologous recombination. In both cases, they contain a selectable marker gene flanked by DNA sequences (about 1 kb each) up- and downstream the target locus. A total of more than 20 genes have recently been disrupted using both methods described here, although only four disruptions have already been published (Ocampo et al. 2012; Lee et al. 2013). The advantageous locus used so far for targeted integration of genes in *M. circinelloides* is the *carRP–carB* locus (Velayos et al. 2000a, b) that includes two genes and three enzymatic activities which drive the production of β-carotene from geranyl–geranyl–pyrophosphate (GGPP). Integration at either the *carRP* or *carB* genes by homologous recombination is easily detectable because disruption of any of these genes leads to colonies with a white phenotype, which is clearly different from the yellow phenotype of the recipient strains. *M. circinelloides* is also a good host for heterologous gene expression (Iturriaga et al. 1992): the *carB* and *carRA* genes of *Blakeslea trispora* were expressed in *M. circinelloides* MS8 and MS23 β-carotene-deficient auxotrophs to generate strains of *M. circinelloides* producing higher levels of β-carotene (Rodríguez-Sáiz et al. 2004), and carotenogenic genes from *Paracoccus* sp. N81106 (a marine bacterium) were also introduced into *M. circinelloides* strains to produce β-carotene derivatives with higher economic significance (Papp et al. 2006; Iturriaga et al. 2012) (*see* Table 4.1).

Thus, to date transformation of protoplasts is the method of choice to get a reasonable number of transformants in a single transformation experiment in *M. circinelloides*. Although the changes

introduced in the original procedure to get and transform the protoplasts could be considered minor ones, they have simplified it and increased the frequency of transformation. Here we will describe such modifications (and problems) so as to get good preparation of protoplasts, and the slight differences in the methods depending on the transformation protocol employed: the PEG-mediated method or electroporation (Gutiérrez et al. 2011).

4.2 Materials

1. Suitable strains of *M. circinelloides f. lusitanicus*. Wild type strain CBS 277.49 and mutant R7B were obtained from Prof. D. von Wettstein (Carlsberg Laboratory, Denmark) (*see* Table 4.1 and Note 1).
2. *Streptomyces #6* was kindly provided by Prof. J. Ruiz-Herrera (University of Guanajuato, Mexico), through Dr. J.M. Fernández Ábalos (University of Salamanca, Spain).
3. Suitable plasmids and linear DNA (*see* Table 4.1 and Note 2).
4. YPG and YNB are respectively the rich and minimal standard media used for the growth of *M. circinelloides* (Lasker and Borgia 1980). YPG: 3 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose. YNB: 1.5 g/L ammonium sulfate, 1.5 g/L glutamic acid, 0.5 g/L yeast nitrogen base (w/o ammonium sulfate and amino acids), and 10 g/L glucose. When solid media are required, final agar concentration should be 20 g/L, and for soft agar 10 g/L (*see* Note 3).
5. MMC (Nicolás et al. 2007): 10 g/L casaminoacids, 0.5 g/L yeast nitrogen base (w/o ammonium sulfate and amino acids), and 20 g/L glucose (*see* Notes 3 and 4).
6. YNB/S medium: Add sorbitol to 0.6 M either on liquid or solid YNB media.
7. YPGS, YNBS, and MMCS: Add sorbitol to 0.5 M to the aqueous preparation of YPG, YNB, and MMC before autoclaving.
8. Jeniaux's medium: 0.8 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $Fe_3Cl_3{\cdot}6H_2O$, 0.1 g/L $CaCl_2$, 0.01 g/L $ZnSO_4{\cdot}7H_2O$, 3 g/L yeast extract and 5 g/L glucose (Jeniaux 1966).
9. Oatmeal medium: 20 g/L oatmeal, 50 % full-fat milk and 20 g/L agar (Suárez 1985).
10. "Streptozyme" induction medium: Jeniaux's medium in which glucose has been substituted by *M. circinelloides* cell walls (1 % v/v).
11. SP buffer: 0.1 M sodium phosphate buffer pH 6.5 (*see* Note 5).
12. SM buffer: 0.6 M sorbitol and 10 mM MOPS pH 6.5.
13. SMC buffer: SM buffer and 50 mM $CaCl_2$.
14. PMC buffer: 0.4 M sorbitol, 10 mM MOPS pH 6.5, 50 mM $CaCl_2$, and 40 % PEG_{4000}.
15. PS buffer: 0.5 M sorbitol and 10 mM SP buffer pH 6.5.
16. D-(+)-Glucosamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA).
17. Chitosan (Sigma-Aldrich, St. Louis, MO, USA).
18. Sorbitol (Sigma-Aldrich, St. Louis, MO, USA).
19. LE (lysing enzymes from *Trichoderma* sp.) (Sigma-Aldrich, St. Louis, MO, USA).
20. RD chitosanase from *Bacillus subtilis* (USBiological, Swampscott, MA, USA).
21. Acid-washed Ballotini glass beads (0.5–1 mm diameter) (*see* Note 6) and a Braun MSK homogenizer coupled to a liquid CO_2 cooler.
22. Amicon concentration apparatus with PM 10 membrane (exclusion limit 10 kDa), together with a magnetic stirrer and a source of N_2 to force the liquid pass through the membrane.
23. Bio-Rad Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA).
24. Gene Pulser Cuvettes (0.2-cm electrode gap) (Bio-Rad, Hercules, CA, USA).

4.3 Methods

During these years, there have been some problems to improve the *M. circinelloides* transformation procedure, mainly due to the enzymes used to disrupt cell walls. The first enzyme used was Novozyme 234 (Novo Industries, Denmark)

which was an extract of *Trichoderma* sp. with high chitosanase activity. After a while, this commercial enzyme disappeared from the market. Fortunately, this gap was filled with LE enzymes (a similar preparation from Sigma-Aldrich) and later, RD chitosanase from *Bacillus subtilis*. Nowadays, the company USBiological Life Science sells a different brand of RD chitosanase isolated from *Streptomyces* sp. N174. In the experiments described below, the RD chitosanase from *B. subtilis* was used. None of the above mentioned enzymes are able alone to produce a satisfactory preparation of *M. circinelloides* protoplasts (except for Novozyme 234), but all of them work very well in combination. The LE/RD enzyme mixture has been the perfect choice to get readily good protoplasts in the labs working with *M. circinelloides* during the last 10–12 years. When Novozyme 234 disappeared from the market, some of us returned to early experiments in which we prepared our own chitosanases from *Streptomyces #6*. Today, home-made "streptozyme" (*see* Note 7) together with LE enzymes from Sigma-Aldrich give an excellent source of *M. circinelloides* protoplasts for the PEG-mediated method. We will describe below how to get large batches of "streptozyme" and its use in preparing and transforming protoplasts of *M. circinelloides*.

4.3.1 *M. circinelloides* Cell-Wall Isolation

1. Inoculate 1 L of YPG liquid medium in a 5 L flask with 10^5 spores/mL of wild type *M. circinelloides* CBS 277.49 strain.
2. Grow the culture for 24–36 h at 28 °C and 180 rpm. Harvest the mycelium by paper filtration and wash it several times with distilled water. Eliminate excess water between several filter paper towels.
3. To approximately every 10 g (wet-weight) of mycelium add 20 g of Ballotini glass beads and 30 mL of sterile distilled water. Mix well and use an appropriate plastic container to disrupt the mycelia in a Braun MSK homogenizer (three times, 1 min each) (*see* Note 8).
4. Collect all samples and eliminate the glass beads by sedimentation (5–15 min, on ice). Wash the glass beads with minimum sterile distilled water and add it to the sample.
5. Centrifuge the glass-free sample in 150 mL bottles at 2,000 × *g* for 10 min. Discard the supernatant and wash the precipitate with distilled water. Repeat the process five times.
6. Mix the precipitates in one or two 150 mL bottles and resuspend in 100 mL of 10 mM NaOH. Incubate at 65 °C for 30 min (*see* Note 9).
7. Wash the cell walls several times with distilled water by centrifuging at 1,000 × *g* for 10 min.
8. When the supernatant is perfectly transparent, collect the cell walls in 200 mL distilled water. Autoclave and reserve (*see* Note 10).

4.3.2 Preparation and Quantitation of "Streptozyme"

Preparation of "streptozyme" is similar to that described by Price and Stork (1975), but later modified by substituting the carbon source in the medium with *Phycomyces blakesleeanus* purified cell walls (Suárez 1985). Instead, we use here *M. circinelloides* cell walls.

One unit of "streptozyme" activity is defined as the amount of enzyme which liberates 1 nmol of glucosamine (or equivalent reducing sugar) per min at 25 °C and pH 6.5. The amount of reducing sugar in the samples is determined by the Somogyi-Nelson method using D-GLUCOSAMINE as a standard.

1. Several days before starting the process, plate a single fresh oatmeal medium Petri dish with an aliquot of stored *Streptomyces #6* and incubate for 3–4 days at 28 °C (*see* Note 11).
2. Recover the cells by washing the plate with sterile distilled water and use some of this to inoculate 100 mL of Jeaniaux's liquid medium. Cultivate for about 24 h at 28 °C and 200 rpm (until it reaches the log phase).

3. Add 2 % (v/v) of the starting inoculum to 2 L of Jeniaux's medium in a 5 L flask. Incubate at 28 °C and 200 rpm for 36–48 h (until late stationary phase).
4. Collect the cells by centrifugation (5,000 rpm/10 min/10 °C) in 150 mL bottles.
5. Add the cells to 1 L "streptozyme" induction medium and incubate at 28 °C and 200 rpm for 14–18 h (*see* Note 12).
6. During the growth of *Streptomyces #6* in the induction medium, take 5 mL aliquots every 30 min after the first 12 h and measure enzyme activity. When the enzyme activity is optimal (typically 16 h), collect the supernatant (*see* Notes 12 and 16).
7. Centrifuge (5,000 rpm/30 min/10 °C) to separate the cells from the supernatant.
8. Concentrate the supernatant to approximately 100 mL by passing it through an Amicon apparatus with a PM 10 membrane (exclusion limit 10 kDa) under N_2 pressure (*see* Note 13).
9. Dialyze against 20 mM sodium phosphate buffer pH 6.5 at 4 °C for 24 h with 2–3 changes of buffer.
10. Make 1 mL aliquots and store at −20 °C (*see* Note 14).
11. Prepare a standard curve by measuring the absorbance at 520 nm of different concentrations of D-glucosamine in 10 mM phosphate buffer pH 6.5 at 25 °C.
12. Prepare a 1 % chitosan solution by homogenizing it in 80 mL of 2 % acetic acid with pestle and mortar. Adjust pH to 6.5 using 1 N NaOH and complete the volume to 100 mL with sterile distilled water (*see* Note 15).
13. To determine enzyme activity, mix 1 mL of homogenized 1 % chitosan solution with 1 mL of sample, and incubate at 25 °C for 10 min. Stop the reaction by heating at 100 °C for 10 min.
14. Centrifuge the samples and carry out the Somogyi-Nelson reactions. Measure the absorbance at 25 °C and 520 nm. Compare the absorbance with the standards and be sure your results are in the linear region of the standard curve.

4.3.3 Preparation and Transformation of *M. circinelloides* Protoplasts by the PEG-Mediated Method

1. Inoculate 10 mL of YPG medium, appropriately supplemented, with 10^8 spores of the recipient strain in a 100 mL flask. Let it stand at room temperature for 2–4 h. Then maintain it overnight at 4 °C (*see* Note 17).
2. Incubate the spores at 28 °C and 180 rpm for 3–4 h. Take samples every 15 min after the first 3 h and observe them under a phase-contrast microscope. If necessary, extend the incubation time until the germ tubes are approximately 5–10 times the length of the spores (*see* Note 18).
3. Wash the germlings twice in 10 mM phosphate buffer pH 6.5. Resuspend in an appropriate volume of the same buffer (see below).
4. Set up a protoplast assay in a 100 mL flask (final concentrations for 20 mL): 0.6 M sorbitol, 10 mM sodium phosphate buffer pH 6.5, 0.5 mg/mL LE enzymes, 4–20 U/mL "streptozyme", germlings and water.
5. Incubate at 30 °C and 60 rpm for 2–3 h. Take samples every 15 min after the first 2 h and observe the loss of cell walls and/or the release of protoplasts under a phase-contrast microscope. If necessary leave the reaction overnight (*see* Notes 18 and 19).
6. When the reaction is complete, recover the protoplasts by centrifugation at $90 \times g$ for 5 min at 10 °C. Wash the protoplasts twice with SM buffer and once with SMC buffer. Resuspend them in 1 mL SMC.
7. Use the protoplasts immediately for transformation or keep them at 4 °C for up to 24 h (*see* Note 20).
8. A typical transformation experiment is carried out with 200 µL of this protoplast stock. To do this, add 10 µL of DNA (0.1–10 µg of DNA) and 20 µL of PMC. Mix well by inversion and incubate on ice for 30 min.
9. Add 2.5 mL of PMC, mix well, and incubate at room temperature for 25 min.

10. Wash the mixture in a large volume of SM twice to remove PEG and resuspend in 2 mL of SM in a 50 mL capped polypropylene tube.
11. To this tube add 50 mL YNB/S soft agar (previously warmed to 45 °C and appropriately supplemented). Mix well and quickly, and pour immediately onto 5–10 plates of YNB/S selective medium.
12. Let the plates stand for several minutes at room temperature and then incubate for 5–10 days at 28 °C (*see* Note 21).

4.3.4 Preparation and Transformation of *M. circinelloides* Protoplasts by Electroporation

1. Collect fresh spores (no more than 1 week old) and resuspend them in YPG medium pH 4.5. Adjust the final spore concentration to 10^7 spores/mL.
2. Incubate overnight at 4 °C, without shaking.
3. Incubate the spores at 26 °C with shaking (300 rpm), until germ tube length becomes about four times the swollen spore diameter. This usually takes 3–4 h.
4. Wash the cells twice by centrifugation in PS buffer pH 6.5 at 340 × *g* for 5 min.
5. Resuspend the pellet in 4 mL of PS buffer. Transfer the germinated spore solution to a 50-mL Erlenmeyer flask.
6. Add 5 mg of LE lysing enzymes dissolved in 1 mL PS buffer, and 100 μL (0.15 U) of Chitosanase RD (dissolved in PS buffer). Incubate at 30 °C with gentle shaking (60 rpm) for about 90 min.
7. Transfer the 5 mL solution to a screw-capped centrifuge tube and fill the tube with cold 0.5 M sorbitol. Wash twice by centrifugation in cold 0.5 M sorbitol at 91 × *g* for 5 min.
8. Resuspend the pellet gently in 800 μL of cold 0.5 M sorbitol. This 800 μL solution allows for eight different transformation experiments.
9. Each tube of transformation mixture must contain 100 μL protoplast solution and 10 μL DNA sample (1 μg total DNA for circular plasmid or 3 μg total DNA for linear fragments; DNA must be dissolved in double-distilled water). Use as a negative control 10 μL of double-distilled water instead of DNA (*see* Note 2).
10. Mix and transfer to the electroporation cuvette.
11. Apply an electrical pulse using the following conditions: field strength of 0.8 kV, capacitance of 25 μF, and constant resistance of 400 Ω.
12. Immediately after the pulse, remove the cuvette and add 1 mL cold YPGS pH 4.5. Keep on ice until all cuvettes have been pulsed.
13. Transfer the liquid of each cuvette to 1.5-mL microcentrifuge tubes.
14. Incubate for 1 h at 26 °C and 100 rpm.
15. Centrifuge at 91 × *g* for 5 min and gently resuspend the pellet in a final volume of 400–600 μL YNBS pH 4.5.
16. Inoculate plates of the adequate selective medium containing 0.5 M sorbitol with 200 μL of transformed protoplasts.
17. Incubate in the dark at 26 °C for 3–5 days (*see* Note 21).

4.4 Notes

1. *M. circinelloides f. lusitanicus* strains CBS277.49 and R7B have been indistinctly used as standards of this organism. The source of genomic DNA, RNA, or cell walls has always been from one or another. During a long time there has been some controversy with strains *M. circinelloides* CBS277.49 and *Mucor racemosus* ATCC 1216b which were considered synonyms. Although Schipper already detected this disagreement in 1976, the mistake continued for years (*see* Wolff et al. 2002). Finally, Díaz-Mínguez et al. 1999, showed that the two strains are different, and in fact, belong to opposite mating types. The complete genome of *M. circinelloides* is available at http://genome.jgi.doe.gov/Mucci2/Mucci2.home.html.
2. Plasmids (*see* Table 4.1). Linear DNA can be isolated from the corresponding plasmids, or these can be used directly for transformation,

provided the DNA of interest is separated from the plasmid core by digestion with two or more different restriction enzymes to avoid re-circularization.

3. Adjust pH to 4.5 for normal, and 3.2 for colonial growth with either 1 M HCl or 1 M NaOH before autoclaving. To avoid hydrolysis of agar during autoclaving when preparing solid media, double-strength solutions of agar and of the other media are autoclaved separately, and mixed after cooling to 50 °C. Any extra-constituent is always added to the liquid solution and the pH is then adjusted. Media are supplemented with 20 μg/mL leucine and/or 200 μg/mL uracil when required. Thiamine and niacin are always added at a 10 μg/mL final concentration after autoclaving.
4. Early observations when using the LeuA or PyrG markers during transformation experiments showed that the number of Leu^+ transformants was higher than $PyrG^+$ or $PyrF^+$ ones in the same conditions. MMCS media solved this question. When Leu^+ transformants are selected, the selective medium must be YNBS (YNB/S) pH 3.2, whereas when $PyrG^+$ or $PyrF^+$ transformants are to be selected, MMCS pH 3.2 is the medium of choice because it renders more transformants than using YNBS (YNB/S) pH 3.2.
5. Dissolve 1.42 g of Na_2HPO_4 in a final volume of 100 mL double-distilled water (0.1 M, solution 1); dissolve 1.38 g of NaH_2PO_4 monohydrate in a final volume of 100 mL double-distilled water (0.1 M, solution 2). Pour solution 1 slowly over 100 mL of solution 2 until pH 6.5 is reached (about 53 mL).
6. To be acid-washed, the Ballotini glass beads are placed in a 2 L flask with up to 300 mL 50 % hydrochloric acid. The flask is then covered with aluminum foil, and the glass beads are stirred at about 75–80 °C for 2 h. Be careful with the outbursts produced by the glass beads and the hot acid. Let the mix cool to room temperature and then discard the acid properly. Rinse the glass beads thoroughly with water until the pH is approximately 7.0. Transfer the beads to clean glass bottles, autoclave, and dry in an oven.
7. "Streptozyme" was the colloquial name given in 1990s to define the extract of enzymes from *Streptomyces #6*. Today, the word Streptozyme is recognized as the name of a commercial test to detect antibodies against several streptococcal enzymes. That is the reason why we use this word with quotation marks throughout the chapter.
8. It is important to maintain the cells cooled when they are being disrupted. Even though the MSK homogenizer is coupled to a CO_2 liquid source, the temperature inside the bottles rises easily. Treat the first sample for 1 min, place it on ice, and then treat a second one. Do the same with the rest of the bottles to complete three rounds of homogenization with each one.
9. NaOH treatment eliminates great part of the proteins and nucleic acids, increasing the presence of polysaccharides in the samples.
10. Cell walls are stable for years at 4 °C and can be autoclaved every time the bottle is opened. A single cell-wall preparation permits up to 20 "streptozyme" inductions.
11. Never try to use directly aliquots of frozen or stored cells of *Streptomyces #6* to start the process. The final enzyme activity decays dramatically. A preliminary growth on the extra-rich oatmeal medium probably induces the ability to produce a higher diversity of (not determined) extracellular enzymes than synthetic media.
12. From the first 12 h on, it is important to establish the enzymatic activity in the supernatant of the growing culture. There are differences from batch to batch.
13. Set the apparatus in a cold room. The filtration takes several hours (4–10) depending on the batch and the membrane age. After the process, wash the membrane in 20 mM NaOH for several hours at room temperature rubbing it softly with gloves from time to time. Never let it dry. Wash thoroughly with distilled water and keep it at 4 °C in 20 mM phosphate buffer pH 6.5 until needed again.
14. "Streptozyme" aliquots can be conserved for years at −20 °C. Since every preparation of "streptozyme" yields about 100 mL, and that in every protoplast assay, 0.1 mL more or less are used (*see* Note 16), we have enough

enzyme preparation for a thousand assays. In fact, we are currently using aliquots prepared in 1990s.

15. Chitosan does not completely dissolve in this solution. Some lumps remain and must be avoided in the enzyme activity determinations. Otherwise, some deviations can occur.
16. Enzymatic activity must be determined during the growth of the culture and in the concentrate. It may differ from batch to batch, but it is routinely between 50 and 200 U/mL in the final aliquots. Nonetheless, an empirical test must be done to know how many units of every batch work well in a protoplast-forming assay. In our hands, 4–12 U/mL of "streptozyme" are enough for a single protoplast assay.
17. Maintaining the spores at room temperature induces their water swallowing and prepares them to germinate. The additional 4 °C treatment overnight synchronizes the germination of the culture.
18. In the early days, it was thought that chitosanase attacked just the growing tip, so the length of the germ tube, when recovering the cells to prepare protoplasts, was limited to about 0.5–2 times the spore size. We have observed that with more time and a little bit less enzyme concentration, the cell-wall degradation occurs throughout the hyphae. This means that we routinely obtain more protoplasts from one initial spore, that they are smaller (they contain fewer nuclei than the original spore), and that they are more resistant to manipulation. So, from the initial 10^8 spores we obtain about 10^7–10^8 viable protoplasts.
19. The protoplast-forming assay can be maintained even overnight without loss of viability and with an increase of protoplasts in the sample.
20. Protoplasts can be maintained at 4 °C without apparent loss of viability. We have observed a slight increase in transformability after 24 h. In an attempt to preserve protoplasts stocks, we kept them in sorbitol-containing buffers at −20 and −80 °C. After several weeks, they were viable, but their transformability was reduced to zero under the same conditions of the previous experiment.
21. Initial transformants in each transformation experiment are always heterokaryons due to the multinucleate nature of spores and protoplasts. These transformants must be grown in selective medium for several vegetative cycles to increase the proportion of transformed nuclei. Identification of homokaryons is differently achieved in gene disruption and targeted gene integration. In the case of gene disruption, transformants that increase the proportion of spores able to grow on selective medium after each vegetative cycle are selected. It is recommended to carry out a rapid PCR amplification (Torres-Martínez et al. 2012), using primers that specifically amplify the disruption, from mycelium of transformants producing more than 50 % spores able to grow on selective medium. Enrichment of transformed nuclei is continued only with those transformants showing a successful gene replacement. In targeted integration of a gene in the *carRP–carB* locus, transformants producing white colonies on selective medium are selected. In both cases, homokaryotic transformants showing 100 % spores able to grow in selective medium (and/or retain the white phenotype), are usually obtained after 2–4 vegetative cycles. Southern-blot analyses or PCR with specific primers should be carried out to confirm either disruption or targeted integration and homokaryosis.

4.5 Final Remarks

M. circinelloides today is a fungal model organism. It is now being used to investigate many biological questions, applied in biotechnological research, and its complete genomic sequence is available. None of these aspects would have been possible without an efficient transformation system. Protoplast-mediated transformation has been shown to be the most effective one for this fungus. Table 4.2 shows a brief summary of some *M. circinelloides* transformation experiments done during the last 25 years. Although the transformation rate is reduced with the

Table 4.2 PEG-mediated vs. electroporation of *Mucor circinelloides f. lusitanicus* protoplasts

Recipient strain	Plasmid	Genetic marker	Treatment	DNA amount (μg)	Topology of plasmid	Number of transformants (T)	Transformation rate (T/μg)	References
R7B	pLeu4	LeuA	PEG	0.1	*Circular*	26	260	Iturriaga et al. (1992)
				1		101	101	
				5		306	61.2	
R7B	pLeu4	LeuA	Electroporation	0.1	*Circular*	33	330	Gutiérrez et al. (2011)
				1		129	129	
				4		259	64.75	
MS12	pEPM9	PyrG	PEG	0.1	*Circular*	15	150	Benito et al. (1995)
				1		88	88	
				5		208	41.6	
MS41	pAVB20	PyrF	PEG	0.1	*Circular*	20	200	Velayos et al. (1998)
				1		181	181	
				5		404	80.8	
MU402	pMAT1554	PyrG	Electroporation	0.1	*Circular*	53	530.0	Gutiérrez et al. (2011)
				1		381	381.0	
				2		505	252.5	
MS12	pSBpyrG4	PyrG	PEG	0.1	*Linearized*	0	0	E.A. Iturriaga (unpublished data)
				1		0	0	
				5		3	0.6	
				10		23	2.3	
MU402	pMAT1554	PyrG	Electroporation	0.1	*Linearized*	0	0.0	Gutiérrez et al. (2011)
				1		0	0.0	
				3.5		4	1.1	
MU520	pMAT1357	PyrG	Electroporation	1	*Circular*	70	70	V. Garre (unpublished data)
	pMAT1357			1	*Linearized*	1	1	
	pMAT1356			1	*Linearized*	2	2	

increase of transforming DNA, the number of transformants in a single experiment grows, indicating that we have not reached a plateau. Transformation efficiency has been improved by modifying the media when using the PyrG or PyrF genetic markers (*see* Note 4). Although electroporation or PEG-mediated transformations give quite similar results, the simplification of these processes allow a more useful application of this technique.

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Transformation of *Saccharomyces cerevisiae*: Spheroplast Method

5

Shigeyuki Kawai and Kousaku Murata

5.1 Introduction

In 1919, Giaja prepared spheroplasts of a *Saccharomyces* species using the digestive juice prepared from a snail (Giaja 1919). Spheroplasts are live cells that have no cell wall and are surrounded by a cell membrane. They are unstable and lyse easily in response to small changes in osmotic pressure or in the presence of detergents (Hutchison and Hartwell 1967). In order to keep spheroplasts intact, they must be maintained in the osmotic stabilizer, usually in ~0.6 M sucrose or ~1.0 M sorbitol. Spheroplasts have been used as models in studies of the permeability of compounds across cell membranes. Spheroplasts have also been utilized for the preparation of intracellular organelles such as mitochondria and vacuoles. Although spheroplasts of *Saccharomyces cerevisiae* exhibit no mating responses and cannot proliferate by budding, they still possess the fundamental abilities of yeast cells; therefore, when embedded in solid (agar) nutrient medium, they can synthesize cell wall components and regenerate into normal cells.

Studies conducted in the latter half of the 1970s revealed that the fusion of plant spheroplasts is significantly accelerated in the presence of polyethylene glycol (PEG). Ferenczy and Maraz (1977) confirmed that the spheroplasts of *S. cerevisiae* can also fuse at high frequency in the presence of PEG. Spheroplast fusion in the presence of PEG was initially applied to the breeding of polyploids or the introduction of organelles into yeast cells. For example, in the presence of PEG, mitochondria isolated from ρ^+ cells of *S. cerevisiae* can fuse with spheroplasts of ρ^0 cells to yield ρ^+ fusants (Ferenczy and Maraz 1977).

Prior to the observation of the effect of PEG on spheroplast fusion by Ferenczy and Maraz (1977), Oppenoorth (1962) reported the transformation of raffinose (RFI)-nonfermentative *Saccharomyces chevalieri* cells to RFI-fermentative cells by incubation of spheroplasts of RFI-nonfermentative cells with DNA fragments prepared from RFI-fermentative cells. Although the precise mechanism was not clear, this was the first report of yeast transformation using spheroplasts and naked DNA. Subsequently, Russell and Stewart (1979) also observed that, in the presence of PEG, maltotriose (MTT)-nonfermentative *Saccharomyces* sp. cells were transformed into MTT-fermentative cells when spheroplasts of MTT-nonfermentative cells were incubated with DNA fragments prepared from MTT-fermentative cells.

A more direct and reliable transformation by exogenous DNA was developed by Hinnen et al. (1978). To obtain definitive evidence of transformation, they used spheroplasts of *S. cerevisiae* AH22

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cells (a *leu2-3 leu2-112*, *his 4-519 can1*) carrying double mutations, which suppressed appearance of revertants (i.e., non-transformed cells), and a YIp-type plasmid DNA, which allowed insertion of exogenous DNA into chromosomal DNA, as confirmed by Southern-blot analysis. Although the transformation frequency was very low, 1–10 transformants/μg DNA, this method opened the way to the use *S. cerevisiae* as a genetic host for molecular-biological research. Following the development of yeast host-vector systems (Beggs 1978, Burgers and Percival 1987, Struhl et al. 1979), high-frequency transformation systems using spheroplasts of *S. cerevisiae* had been successfully achieved.

However, the spheroplast method has some disadvantages, as follows:

1. Spheroplast preparation is tedious and complex.
2. Spheroplasts are unstable and form clots by aggregation, which decreases the efficiency of DNA uptake.
3. The frequency with which spheroplasts regenerate to normal cells is low.
4. The efficiency of DNA uptake by spheroplasts is low.
5. Fusants are occasionally formed.
6. A complex process and a long period of time (more than 1 week) are required.
7. Selection of transformants in the presence of antibiotics is not easy, because spheroplasts are usually susceptible to antibiotics.
8. Replica plating is impossible.

These disadvantages inherent to the spheroplast method were overcome by the establishment of monovalent cation-dependent transformation method using intact cells of *S. cerevisiae* (Ito et al. 1983). However, the spheroplast method is still useful for special cases, e.g., transformation with yeast artificial chromosomes with lengths of 100–1,000 kb, synthetic bacterial genomes cloned in yeast vectors, or infectious prion particles (Benders 2012, Burke et al. 1987, King et al. 2006).

Below, we describe the spheroplast method for transformation of *S. cerevisiae* that achieved the highest transformation efficiency (Burgers and Percival 1987), as reviewed previously (Kawai et al. 2010). The outline of the method is depicted in Fig. 5.1. The method consists of five steps: (1) spheroplast preparation, (2) incubation of spheroplasts with DNA in the presence of PEG and Ca^{2+}, (3) embedding into solid medium, (4) regeneration of the cell wall, and (5) selection of transformants. It is noteworthy that, in contrast to the case of *S. cerevisiae*, spheroplasts prepared from the cells of *Schizosaccharomyces* species can proliferate in liquid medium.

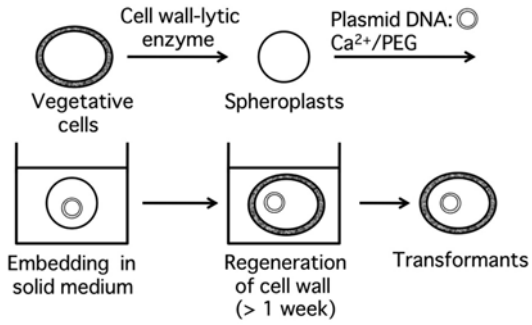

Fig. 5.1 Transformation process using spheroplasts. All procedures are performed in the presence of an osmotic stabilizer

5.2 Spheroplast Method

5.2.1 Reagents

All autoclaving steps are done at 121 °C for 20 min.

1. Liquid YPD medium: 1.0 % yeast extract, 2.0 % tryptone, and 2.0 % glucose in pure water (pH 5.6). For solid medium, add 2.0 % agar. Autoclave.
2. SCEM: 1 M sorbitol, 0.1 M sodium citrate (pH 5.8), 10 mM EDTA, and 30 mM 2-mercaptoethanol (2-ME). Add 2-ME after autoclaving the other components.
3. STC: 1 M sorbitol, 10 mM Tris–HCl (pH 7.5), and 10 mM $CaC1_2$. Autoclave.
4. 20 % PEG: 10 mM Tris–HCl (pH 7.5), 10 mM $CaC1_2$, and 20 % w/v PEG 8000. Filter sterilize.
5. SOS: 1 M sorbitol, 6.5 mM $CaC1_2$, 0.25 % yeast extract, and 0.5 % Bacto™ peptone. Filter sterilize.
6. TOP: 1 M sorbitol and 2.5 % agar in 0.67 % yeast nitrogen base w/o amino acids (Becton Dickinson and Company), with appropriate amino acids and uracil, and 2 % w/v glucose. Autoclave.

7. SORB plates: 0.67 % yeast nitrogen base w/o amino acids, with appropriate amino acids and uracil, 3.0 % w/v glucose, 0.9 M sorbitol, and 2 % w/v agar. Autoclave.

5.2.2 Procedures

Centrifuge cells and spheroplasts at 400–600 g and 200–300 g, respectively.

1. Grow the cells overnight with vigorous aeration in 50 ml of YPD to a concentration of about 3×10^7 cells/ml. Harvest by centrifugation.
2. Wash the cells successively with 20 ml of sterile water and 20 ml of 1 M sorbitol by resuspension followed by 5-min spins. Resuspend cells in 20 ml of SCEM, add 1,000 U of lyticase (or Zymolyase, a cocktail of various cell wall–lytic enzymes), and incubate at 30 °C with occasional inversion.
3. To monitor the extent of spheroplasting, measure the decrease in the OD_{600} of a 10-fold dilution of spheroplasts in water. When ~90 % of cells have become spheroplasts (~15–20 min), harvest the spheroplasts by centrifugation for 3–4 min.
4. Gently resuspend the spheroplasts in 20 ml of 1 M sorbitol using a 1-ml pipette, and then centrifuge for 3–4 min. Gently resuspend the pellet in 20 ml of STC, and centrifuge again for 3–4 min. Resuspend this pellet in 2 ml of STC.
5. Mix 100-µl aliquots of spheroplasts in STC with plasmid DNA and calf thymus or *E. coli* carrier DNA (total of 5 µg of DNA in <10 µl).
6. After 10 min at room temperature, add 1 ml of 20 % PEG, gently resuspend the spheroplasts, and incubate for another 10 min. Centrifuge the spheroplasts for 4 min.
7. Resuspend the pellet in 150 µl of SOS, and incubate at 30 °C for 20–40 min. Dilutions of the spheroplasts should be made in the same medium.
8. Add 8 ml of TOP, kept at 45–46 °C. Invert the tube quickly several times to mix, and then plate the suspension immediately on selective SORB plates. The plates were incubated at 30 °C for around 1 week.
9. Pick up transformants or destroy the agar plates to obtain transformants when picking up the transformants is difficult.

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Part III

Transformation Methods: Electroporation

Electroporation Mediated DNA Transformation of Filamentous Fungi

6

B.N. Chakraborty

6.1 Introduction

Recombinant DNA technology offers the potential to make well-defined alterations on the genetic make-up of an organism. The development of this technology was based on two advances: the ability to splice together defined fragments of DNA and the development of appropriate vectors that are able to introduce, replicate, and express recombinant DNA in a given organism. The consequence was the ability to make specific genetic modifications of an organism. The technology provides powerful tools to further understanding of genes, gene regulation, and genomic organization.

The first successful use of recombinant DNA techniques employed prokaryotes (most notably *Escherichia coli*). However, it was immediately apparent that the techniques had great potential for approaching numerous biological, biochemical, and genetic questions in many systems. Many organisms have unique properties that could be studied and exploited if techniques could be developed for the transformation of these organisms.

The techniques of molecular biology have opened new avenues of research with filamentous fungi. Filamentous fungi particularly *Aspergillus nidulans* and *Neurospora crassa* have proven to be extremely useful model systems for investigations of eukaryotic gene structure, organization, and regulation of expression. Cloning of specific structural and regulatory genes, utilizing recombinant DNA technology has facilitated analyses of genetic regulation at the molecular level. A critical prerequisite for such studies is the availability of a suitable transformation system. Hinnen et al. (1978) developed the technique to transform *Saccharomyces cerevisiae* using auxotrophic markers and also *E. coli* shuttle vectors for this organism. Techniques for manipulation of DNA in model filamentous fungi (*N. crassa* and *A. nidulans*) were developed shortly thereafter (Case et al. 1979; Ballance et al. 1983; Yelton et al. 1984). An efficient transformation procedure for *N. crassa* using protoplasts in which a non-reverting recipient strain, deficient in the catabolic as well as the biosynthetic dehydroquinase (qa-2 aro-9), was transformed with a recombinant *E. coli* plasmid, harboring the *N. crassa* qa-2^+ gene, encoding the catabolic dehydroquinate hydrolase (EC 4.2.1.10). This method has been employed successfully with several species of filamentous fungi (Fincham 1989).

Progress in research on the molecular genetics of filamentous fungi has required concurrent development of techniques for introduction of genes (transformation), vectors for carrying in the DNA, and isolation of fungal genes. Most of the molecular studies have concentrated on the ascomycetous fungi, *N. crassa* and *A. nidulans*

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for which there has been extensive information provided by classical genetic analysis complemented with molecular approaches. Genetic transformation methods of filamentous fungi can be classified into two major types viz. biological and physical. *Agrobacterium tumefacians* mediated DNA transformation (Michielse et al. 2005) and preparation of protoplasts from fungal cells using cell-wall degrading enzymes are the common methods for transformation under biological types (Rossier et al. 1985). In order to improve the cell wall permeability to DNA as well as without forming protoplast, high concentration of lithium ions (Dhawale et al. 1984) or calcium ions (Neumann et al. 1996) have been used for successful transformation. However, genetic transformation of fungi based on physical methods such as electroporation (Charaborty and Kapoor 1990), biolistics (Rivera et al. 2012), agitation with glass beads (Gurpilharesa et al. 2006; Singh and Rajam 2013), vacuum infiltration (Bechtold et al. 1993), and shock waves (Lauer et al. 1997; Magana-Ortiz et al. 2013) has significantly improved the transformation capacities. An insight into the molecular arrangement of filamentous fungal genes has been made possible with the electroporation mediated DNA transformation systems coupled with standard recombinant DNA technology in filamentous fungi (Charaborty and Kapoor 1990; Chakraborty et al. 1991). Electroporation has become a valuable technique for transfer of nucleic acids into adherent or suspension eukaryotic cells (electrotransfection) and prokaryotic cells (electro-transformation). The technique is an excellent alternative for many cell types which cannot be transfected or transformed by chemical methods. It has been employed successfully to transfer heterologous DNA into microbial cells (Calvin and Hanawalt 1988; Friedler and Wirth 1988; Miller et al. 1988; Powell et al. 1988; Dower et al. 1988; Howard et al. 1988; Mclntyre and Harlander 1989; Wen-Jun and Forde 1989; Theil and Poo 1989; Delorme 1989; Richley et al. 1989; Hatterman and Stacey 1990), plant protoplasts (Shillito et al. 1985; Fromm et al. 1986; Riggs and Bates 1986; Toriyama et al. 1988; Bellini et al. 1989), and animal cells (Zerbib et al. 1985; Narayanan et al. 1986; Toneguzzo and Keating 1986; Toneguzzo et al. 1986; Tur-Kaspa et al. 1986; Chu et al. 1987; Spandidos 1987; Knutson and Yee 1987; Hama-Inaba et al. 1987). Extensive review on the process and strategies for fungal transformation is available (Fincham 1989; May 1992; Riach and Kinghorn 1996; Prasanna and Panda 1997; Ruiz-Diez 2002; Meyer 2008; Rivera et al. 2012; Rivera et al. 2014).

The present document will reflect the process by which transformation systems were developed for a number of filamentous fungi with special emphasis on recent advances in our understanding on the electroporation mediated transformation of filamentous fungi.

6.2 Selection Methods

Two types of selection systems are available for filamentous fungi. The first class involves complementation of an auxotrophic mutation with the matching cloned wild-type gene. Examples include transformation of *trpC* and *argB* auxotrophs of *A. nidulans* (Yelton et al. 1984), complementation of *qa-2* (Case et al. 1979) and *am-1*(Kinnard et al. 1982) auxotrophs of *N. crassa* with homologous genes. The heterologous *N. crassa pyr4* gene has been used to complement the *A. nidulan* pyrG mutant (Ballance et al. 1983). A major limitation to use this type of selection is that it requires to have both a cloned wild-type gene as well as the corresponding mutation in the recipient strain. This latter requirement is not always trivial to satisfy, especially for some on industrially important filamentous fungi. In these organisms, introduction of specific mutations may be difficult or undesirable, and at a minimum, quite time consuming. The alternative method for selection of transformants employs dominant markers (Table 6.1) that can be selected in wild-type recipients.

The dominant resistant markers in fungi include the dominant ß-tubulin genes encoding resistance to benomyl in *N. crassa* (Orbach et al. 1986) and in *Aspergillus niger*. The neomycin phosphotransferase genes from Tn903 and Tn5 encoding resistance to the amino-glycoside antibiotic G418

Table 6.1 Selectable markers used for protoplast transformation in filamentous fungi

Fungi	Species of origin	Marker used	Phenotype(s)
A. nidulans	*A. niger*	oliC	Oligomycin resistance
Aspergillus sp.	*N. crassa*	Pyr-4$^+$	Pyrimidine synthesis
Aspergillus sp.	*E. coli*	lacZ^c	ß-Galactosidase
A. niger	*A. nidulans*	Arg B$^+$	Arginine synthesis
C. heterostrophus	*E. coli*	Hyg B^r	Hygromycin B resistance
C. heterostrophus	*A. nidulans*	amdS$^+$	Acetamide utilization
Colletotrichum trifolii	*E. coli*	Hyg B^r	Hygromycin B resistance
C. trifolii	*N. crassa*	Ben^r	Benomyl resistance
C. trifolii	*A. nidulans*	amdS$^+$	Acetamide utilization
C. acremonium	*E. coli*	Hyg B^r	Hygromycin B resistance
Fulvia fulvum	*E. coli*	Hyg B^r	Hygromycin B resistance
Gaeumannomyces graminis	*N. crassa*	Ben^r	Benomyl resistance
M. grisea	*A. nidulans*	Arg B$^+$	Arginine synthesis
Phycomyces blakesleeanus	*E. coli*	Neo^r	Kanamycin, G418 resistance
P. chrysogenum	*A. nidulans*	amdS$^+$	Acetamide utilization
P. chrysogenum	*N. crassa*	Pyr-4$^+$	Pyrimidine synthesis
S. cerevisiae	*E. coli*	Hyg B^r	Hygromycin B resistance
S. cerevisiae	*E. coli*	bla^c	ß-Lactamase
Septoria nodorum	*E. coli*	Hyg B^r	Hygromycin B resistance
Schizophyllum commune	*E. coli*	Neo^r	Kanamycin, G418 resistance
Ustilago maydis	*E. coli*	Hyg B^r	Hygromycin B resistance
U. maydis	*E. coli*	Neo^r	Kanamycin, G418 resistance

have been used for *Cephalosporium acremonium* and *Penicillium chrysogenum*, resistance to hygromycin B has been used for *C. acremonium* (Queener et al. 1985). In all these cases a fungal promoter is required to obtain adequate levels of expression of the prokaryotic genes. All of these dominant markers have the distinct advantage of not requiring the presence of a particular mutation in the recipient but rather require merely that the organism be sensitive to the applied selective pressure and that the cloned gene can be expressed in the recipient and relieve the selection. Providing that these conditions are met, virtually any fungal species should, in principle be transformable with the selective markers.

6.2.1 Electroporation

While relatively high efficiencies of transformation have been reported using protoplasts, their preparation entails a prolonged procedure involving careful monitoring of the various steps in the protocol. Optimization of the conditions for individual batches of cell wall degrading enzymes is invariably required. In addition, regeneration of protoplast may also present problems. The lithium acetate procedure has not been widely used, probably on account of a low rate of success. In contrast, electroporation offers a relatively simple and rapid technique, avoiding the use of potentially toxic chemicals, such as alkali cations and the necessity for preparation of protoplasts. When a cell is exposed to an electric field, the membrane components become polarized and a voltage potential develops across the membrane. If the potential difference exceeds a threshold level, the membrane breaks down in localized areas and the cell becomes permeable to exogenous molecules (Knight 1981). The induced permeability is reversible, provided the magnitude or duration of the electric field does not exceed a critical limit, otherwise the cell is irreversibly damaged. The use of an electric field to reversibly permeabilize cells has been termed “electroporation”. Although the mechanism of electroporation

is not known, the potential difference required for membrane breakdown has been estimated from 0.3 to approximately 1.5 V and may depend on factors such as membrane composition, temperature, and duration of the electric field (Knight and Scrutton 1986).

The successful application of electroporation to many cell types, both eukaryotic and prokaryotic, suggests that the technique may be universally applicable. Electrical charge can be stored in a capacitor which produces an exponential pulse with a voltage amplitude that approximates the setting on the power supply and is subsequently discharged across a sample. Direct discharge of a power supply yields a pulse similar in shape to an exponential pulse, however, the actual voltage applied to the sample may be much less than the setting on the power supply. Several pulse waveforms are effective in electroporating fungal cells. The exponential waveform is determined by two electrical variables—the peak voltage (V_0) and the pulse length (expressed as the RC time constant, τ). The potential applied across as suspension of cells will be experienced by any single cell as a function of the field strength ($E = V/d$, where d is the distance between the electrodes) and the length of the cell. Because the fungal spores are small in size, they might be expected to require higher field strength for electroporation, and in practice, this seems to be the case. Electric pulses enhance the formation of pores and induce membrane permeabilization providing a local driving force for ionic and molecular transport through the pores. The accepted theory is that exogenous DNA is captured through these transient pores.

A rapid and efficient electroporation procedure for transformation of germinated conidia of a mutant strain of *N. crassa* was developed by Charaborty and Kapoor (1990) and summarized in Fig. 6.1. Using the qa^{2+} gene encoding the catabolic dehydroquinase in conjunction with a double mutant (qa-2 arom9)—deficient in both the biosynthetic and the catabolic dehydroquinase—as a recipient strain, stable transformants were obtained. Initial attempts were made for standardization of the experimental conditions on the effect of varying the field strength and capacitance on cell viability and transformation efficiency. No transformants were detected at 1 μF and 3 μF capacitance setting in conjunction with field strength values ranging from 3.0 to 12.5 KV/cm (using the Biorad Gene Pulser Xcell). Increasing the electric field strength at 12.5 KV/cm and 25 μF capacitance led to a significant decrease (55 %) in cell viability. However, the highest efficiencies were achieved with a field strength of 12.5 KV/cm, a pulse length of 5 m/sec, and 25 μF capacitance. The age of the starting material is a critical factor in determining the yield of transformants. The most suitable age should be determined empirically for each fungal species and strain. For successful transformation of *N. crassa*, conidia after 15 days of culturing were found to be suitable, while for *Penicillium urticae* conidia after 12 days, *Aspergillus oryzae* after 10 days, and pycnidiospores of *Leptosphaeria maculans* after 14 days of culturing were found to be efficient for transformation (Chakraborty et al. 1991). As the cell wall presents a physical barrier for uptake of DNA, methods for weakening the cell wall were evaluated.

Application of heat shock (45 °C, 30 min), polyethylene glycol or DTT had no discernible effect on the uptake of DNA by *N. crassa* cells. Pretreatment with amphotericin B, a polyene antibiotic known to disrupt the selective permeability of cell membrane (Kerridge 1986), also proved to be ineffective. Incubation with β-glucuronidase (Sigma type H-1, from *Helix pomatia*) to germinated conidia, prior to electroporation, proved effective in weakening the cell walls and consequently, in enhancing the transformation efficiency (Table 6.2). Successful genetic transformation by electroporation in various filamentous fungi has been demonstrated (Table 6.3).

The introduction of DNA into cells does not necessarily imply that it will be generally useful as a research tool. However, if the fate of transforming DNA can be predicted, it could be a useful tool in the study of questions of biological interest. This is the case for *S. cerevisiae*, where nearly all plasmids carrying selectable markers integrate primarily by homologous recombina-

Fig. 6.1 Electroporation transformation protocol of *N. crassa* using germinating conidia

tion. However, filamentous fungi differ from *S. cerevisiae* in that high frequency transformation often results from nonhomologous (ectopic) integration of DNA into the genome, as well as homologous integration. Southern blot analysis of restriction endonuclease-digested DNA from a random sample of transformants demonstrated the integration of the plasmid in the genome. Hybridization with ^{32}P-labeled probe showed only the resident qa-2 genes in the untransformed recipient strain (Fig. 6.2a, b: lane 1). In contrast, the analysis of transformants revealed ectopically integrated copies of qa-2^{+} gene (Fig. 6.2a: lanes 2–5; Fig. 6.2b: lanes 2 and 6) in a slight majority; integration events that can be attributed to nonhomologous recombination. Other transformants showed integration of the introduced DNA at the correct locations. Approximately 60 % of the integration events were attributed to nonhomologous recombination in *N. crassa*. The remaining

Table 6.2 Optimal electroporation conditions for transformation of *N. crassa*

Conditions	Untreated	Pretreated germinated conidia with β-glucuronidase (1 mg/ml)
Capacitance (μF)	25.0	25.0
Field strength (KV/cm)[a]	12.5	12.5
Time constant (msec)	5.0	5.0
Number of conidia	5.8×10^6	6.3×10^6
Cell viability (%)[b]	57.0	54.0
Transformation efficiency	5.5	41.0

[a]Field strength of 12.5 KV/cm was generated by directing 2.5 KV of electric discharge from 25 μF capacitor through a pulse controller (set at 200 Ω in parallel with the sample) and then through the cuvette with 0.2 cm electrode gap
[b]Percentage of conidia surviving electroporation treatment
[c]Stable transformants per μg of DNA

Table 6.3 Electroporation protocol established for various fungi and Yeast

Fungi and Yeast	References
Aspergillus awamori	Ward et al. (1989)
Aspergillus fumigatus	Burns et al. (2005)
Aspergillus giganteus	Meyer et al. (2003)
A. nidulans	Sanchez and Aguirre (1996)
A. niger	Ozeki et al. (1994)
A. oryzae	Chakraborty et al. (1991)
Candida albicans	Thompson et al. (1998)
Candida maltose	Kasuske et al. (1992)
Candida tropicalis	Rohrer et al. (1992)
Colletotrichum gloeosporioides	Robinson et al. (1999)
Epichloe typhina	Dombrowski et al. (2011)
Hansenula polymorpha	Faber et al. (1994)
L. maculans	Chakraborty et al. (1991)
Metarhizium anisopliae	Leger et al. (1995)
Mucor circinelloides	Gutierrez et al. (2011)
Mycosphaerella graminicola	Adachi et al. (2002)
N. crassa	Charaborty and Kapoor (1990)
Neurospora spheroplasts	Kothe and Free (1996)
P. urticae	Chakraborty et al. (1991)
Pichia methanolica	Lu et al. (2013)
Pichia pastoris	Wu and Letchworth (2004)
Piriformospora indica	Kumar et al. (2013)
S. cerevisiae	Delorme (1989)
Scedosporium prolificans	Ruiz-Diez and Martinez-Suarez (1999)
S. pombe	Hood and Stachow (1990)
Schwanniomyces occidentalis	Costaglioli et al. (1994)
Trichoderma harzianum	Goldman et al. (1990)
Wangiella dermatitides	Kwon-Chung et al. (1998)
Yamadazyma ohmeri	Piredda and Gaillardin (1994)
Yarrowia lipolytica	Nuttley et al. (1993)

transformants carried the plasmid DNA at homologous sites. Frequently integration of multiple copies of the plasmid was witnessed.

A useful feature of transformation in filamentous fungi is that high frequencies of co-transformation (30–90 %) of nonselected plasmids and high numbers of integrated copies can be obtained. Co-transformation using two circular plasmids—pBsqa, containing the selectable qa^{2+}marker inserted in the Bluescript vector, and pUGX121, containing a fragment of the *N. crassa* hsp70 gene as the co-selected gene—was demonstrated by localization of DNA of both plasmids at ectopic as well as homologous sites in individual transformants (Chakraborty et al. 1991). Kapoor et al. (1993) have also successfully employed electroporation for introduction of plasmids harboring heat shock genes (*hsp*70 and *hsp*80) and the *gdh* gene, encoding the NAD-specific glutamate dehydrogenase of *N. crassa*, into *N. crassa* cells by means of co-transformation with the plasmids containing the *qa-2*$^+$ selectable marker. The high frequency of co-transformation observed has led to the suggestion that there is a subpopulation of cells or nuclei that are especially competent for DNA integration. It is likely that this competence phenomenon contributes to the variation in transformation events seen with different protocols with the same and different organisms. Expression of a human metallothione in gene *mt-IIA*, a member of a multiple-gene family comprising a set of metal-responsive genes, in wild type strain 74A *N. crassa* was also documented (Kapoor et al. 1993).

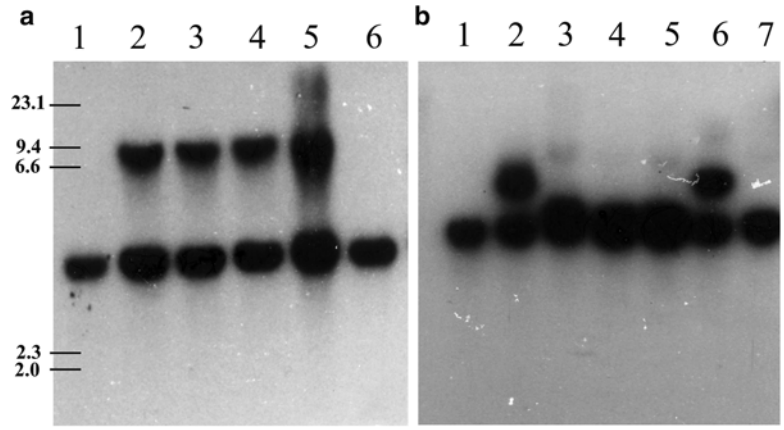

Fig. 6.2 Southern blot analysis of genomic DNA of *N. crassa* transformants digested with (**a**) *Hind*III and (**b**) *Eco*R1 and hybridized with ^{32}P-labeled 2.4-kb *Bam*H1 fragment containing the *N. crassa qa-2*$^{+}$ DNA. (Lane 1) untransformed recipient strain; (lanes 2–7) transformants

6.2.2 Premeiotic Instability: the RIP Effect

DNA sequence duplications provide the critical first step for gene amplification and provide the raw material for evolution of new genes. While vital for evolution, sequence duplications can also have negative consequences. Dispersed repeated genes can mediate exchanges resulting in deletions, inversions, or translocations. In addition, altered gene dosage can result in a detrimental imbalance of gene products. In many organisms, such as fungi and bacteria, virtually all genes are present in one copy per haploid genome.

In the multicellular fungus *N. crassa*, the paucity of duplicated genes may not simply be due to natural selection. Duplications are efficiently detected and altered in specialized (dikaryotic) tissue formed by fertilization (Selker et al. 1987; Selker and Garrett 1988). The process affects both copies of a duplicated sequence as revealed by gene activation, changes in the position of restriction sites, and de novo methylation of cytosines in the repeated DNA. Because the process is limited to the stage between fertilization and nuclear fusion, susceptible cells have a nucleus from each parent. Thus a cell should survive a duplication and inactivation, even in an essential gene, so long as the duplication was in only one of the parents. Both nuclei should deliver their genetic material to meiosis. Standard recombination processes would produce meiotic products having different combinations of altered and unaltered copies of the duplicated sequences. The cells receiving only altered copies may not be viable. Because of the timing of the inactivation and the alteration of restriction site patterns, this process as designated "rearrangement induced premeiotically" (RIP).

Discovery of premeiotic recombination and RIP grew from the development of DNA-mediated transformation in *Neurospora*. Investigation of the fate of transforming sequences in crosses of pES174 transformants led to the discovery of RIP. The first clue came from examining progeny derived from crosses of T-ES174-1, the transformant with the local duplication of the flank region. Normal segregation of the transformation marker, Am+progeny had suffered sequence alterations in both copies of the flank region. Although the overall length of the sequences appeared at least roughly unchanged, the arrangement of restriction sites in the DNA showed numerous alterations. Use of the isoschizomers *Sau*3A, *Mbo*I, and *Dpn*I, produced evidence for both changes in the primary structure of the DNA and extensive *de novo* methylation of cytosines (Selker et al. 1987).

Alterations resulting from RIP were identified by cloning the affected sequences. Restriction analysis of the cloned sequences that had been exposed to RIP revealed novel fragments in the genomic DNA, strengthening the conclusion that the alterations were not simply due to some form of DNA modification such as methylation. To determine whether the cloned sequences, altered by RIP, had suffered any gross rearrangements, hetero duplexes between the altered sequences and their native counterparts were prepared and examined by electron microscopy. Renewed insights led to a name change from "rearrangement induced premeiotically" to "repeat induced point mutation" (Cambareri et al. 1989).

One of the remarkable features of the RIP process is its efficiency. Duplicated sequences are detected at high frequency, whether or not they are genetically linked, and are then scrambled with polarized transition mutations. The same type of mutations occur nonspecifically at lower frequencies in bacterial cells that are deficient for uracil DNA glycosylase, apparently because of spontaneous deamination of cytosines, to give uracils. In normal cells, uracil glycosylase presumably removes uracils from DNA before DNA replication to avert potential mutations. Deaminaton of 5-methylcytosine produces thymine (5-methyluracil), which is not a substrate of uracil glycosylase. Thus, deamination of 5-methylcytosines in DNA would produce G–T mismatches, which if "repaired" to A–T, or resolved by DNA replication, would establish a polarized transition mutation. Indeed, it has been shown in *E. coli* that 5-methylcytosines can be mutational hot spots. The frequency of transition mutations in RIP is too high to be accounted for by spontaneous deamination of cystosines or 5-methylcytosines. Nevertheless, RIP may occur by a related mechanism. Specifically, cytosines or methylcytosines in duplicated sequences may be enzymatically deaminated and simply left unrepaired. Mismatches resulting from such a mechanism would be resolved by DNA replication. The fact that more G–A than C–T changes were observed in one strand in the case of mild sequence alteration by RIP, may reflect this mechanism (Cambareri et al. 1989). The process operates before meiosis in the period between fertilization and nuclear fusion, a stage thought to consist of roughly ten cell divisions.

RIP may be limited to a subset of the filamentous ascomycetes. The process has not been detected in basidiomycetes (Munoz-Rivas et al. 1986; Binninge et al. 1987) and duplicate sequences are not activated in the well-studied yeast *S. cerevisiae* or *Schizosaccharomyces pombe*, neither of which have a heterokaryotic phase in their life cycle. In contrast, duplicate sequences are inactivated in the heterothallic filamentous ascomycete *Ascobolus immersus*, which, like *N. crassa*, has an extended stage in which haploid nuclei of different strains share a common cytoplasm (Goyon and Faugeron 1989; Faugeron et al. 1990). Preliminary evidence suggests that RIP also operates in the plant pathogens *Gibberella fujikuroi* and *Gibberella pulicaris*, both also being heterothallic filamentous ascomycetes with a dikaryotic phase preceding karyogamy. In contrast, RIP does not occur, at least at high frequency, in two other heterothallic filamentous ascomycetes, *Magnaporthe grisea* and *Cochliobolus heterostrophus*, both of which are also plant pathogens (Selker 1990). RIP has not been found in the two homothallic filamentous ascomycetes that have been examined, *Sordaria macrospora*, (Le Chevanton et al. 1989) and *A. nidulans*, nor in the functionally homothallic ascomycetes *Podospora anserine* (Coppin-Raynal et al. 1989). Nevertheless, all three organisms show high frequency deletion of tandem repeates. Considering that RIP inactivates both members of a duplication, it seems reasonable from an evolutionary standpoint that RIP would be limited to homothallic cells in outbreeding organisms. All the nuclei in the premeiotic tissue of a homothallic fungus usually come from a single parent. If that parent happened to have a duplication of an essential gene, RIP might result in cell death.

The fate of transforming sequences in crosses of *N. crassa* transformants (E-26 and E-43) were examined. In these transformants ectopic integration of both qa^{2+} and *hsp70* gene were evident. Tetrad analysis were accomplished either with ordered asci squeezed from perithecia (Fig. 6.3a)

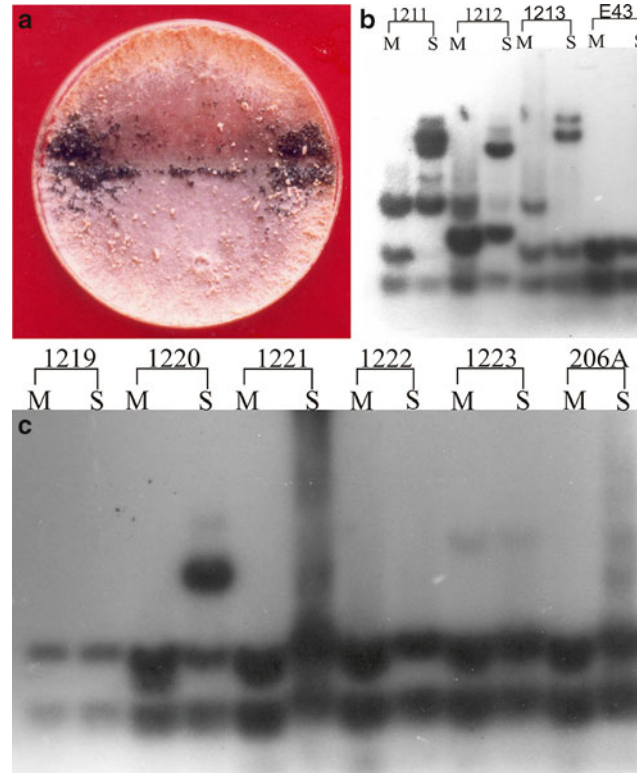

Fig. 6.3 (**a**) Crossing of *N. crassa* wild type (ORSa) and transformant (E-43); (**b**, **c**) disruption of *hsp*-1 DNA of *N. crassa* by RIP. Southern blot of genomic DNA of the transformant (E-43) and randomly picked progeny (1211–1213 and 1219–1223) of the cross E43 × ORSa (wild type), digested with *Mbo*1(M) and *Sau*3A(S) and hybridized with *hsps*-1 DNA. 206A is the original host strain (qa-2 arom-9 double mutant). RIPs are evident in 1211, 1212, 1213, 1220, and 1223

or with asci shot as an unordered group. In either case, germination of the separated spores was usually poor unless the ascospores were allowed to ripen for at least a week, or preferably longer. Corn-meal agar supported good sexual development, without an abundance of conidia.

Greater instability of the transformation marker was observed in crosses of mutants E-26 and E-43 following colony blot hybridization. Randomly selected first generation offspring of these two transformants were tested at the DNA level by southern hybridization to look for physical evidence of RIP. It is evident that plasmid DNA transferred to *N. crassa* cells via transformation integrates predominantly at ectopic sites in the chromosomes, as a result of nonhomologous recombination, thereby producing duplication of DNA sequences. As a consequence of certain premeiotic events, in *N. crassa* the duplicated sequences are prone to inactivation of massive methylation of C residues, followed by deamination leading to GC–AT base pair transitions. These changes are irreversible which provides a powerful method of isolating "functional" deletions (null mutations). Approximately 50 and 60 % of the progeny from crosses of E-26 and E-43 were qa^{2+}, respectively. Southern blot analysis of various progeny strains, digested with *Mbo*I and *Sau*3A both recognizing the sequence GATC, while methylation of the C residue blocks*Sau*3A to cleave at such sites, whereas *MboI* digestion is unaffected (Chakraborty et al. 1995). However, a GC–AT transition will render this sequence unrecognizable by either enzyme. Among the progeny, occurrence of RIPs is evident predominantly in isolates 1211, 1212, 1213, and 1220. Also isolate 1223 demonstrated signals of RIP but less active (Fig. 6.3b, c). When further exposed to heat shock temperature, these strains exhibited a variable range of thermotolerance, suggesting that the development of thermotolerance had been partially compromised.

6.3 Conclusion

The electroporation system described in this communication offers a simple, rapid, and efficient method for transformation of various species of filamentous fungi without the need to produce protoplast or the use of toxic chemicals, thereby opening the door for over one million unexplored fungal species that could potentially benefit from such system. This procedure is directly applicable to sporulating species. However, it can also be adapted for use with mycelia or nonconidiating species. In order to ensure consistently high yields of transformants for pathogenic, ecologically as well as agriculturally important microorganisms it is critical to establish precise experimental conditions such as selection of the age for conidia and germination stage, nature of mycolytic enzyme(s), treatment duration choice of selectable marker(s) as well as selection medium which are critical factors affecting transformation. Multiple copies of plasmids can, and often do, integrate at unlinked sites, thus provide the potential for increased yield of the desired product. Electroporation is rapidly becoming a general method of major importance in cell biology. Repeat induced point mutation (RIP) has proved to be useful in the mutagenesis of specific DNA fragments in vivo, which can be employed to alter the major *hsp* gene to assess its effect on thermotolerance of various species of filamentous fungi. The increasing availability of genomic information which provides potential for innovation to identify new promoters and regulatory sequences in order to enhance heterologous gene expression and the application of genetic transformation promises a bright future for fungal biotechnology.

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Chemical Transformation of *Candida albicans*

7

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7.1 Introduction

For years, the opportunist pathogenic yeast *Candida albicans* has been described as an obligate diploid, with no complete sexual cycle. Consequently, most of the molecular knowledge on this organism has been gained through reverse genetics approaches taking advantage of genetic transformation methods. Even though the recent isolation of haploid strains of *C. albicans* (Hickman et al. 2013) may facilitate the development of forward genetics (possibly hampered by spontaneous diploidization associated with a high fitness cost), genetic transformation is deemed to remain the method of choice to study gene function in both haploid and diploid *C. albicans* strains.

Most genetic engineering approaches in *C. albicans* are based on integrative transformation. Indeed, episomal DNA is most often instable in *C. albicans* and, although autonomously replicative plasmids have been developed (Kurtz et al. 1987; Pla et al. 1995), they have been abandoned in favor of vectors allowing targeted integrative transformation. These vectors include the widely used CIp10 vector and its derivatives which target the *RPS1* locus (Murad et al. 2000). Another set of vectors targeting an intergenic region on chromosome 5 has recently been constructed (Gerami-Nejad et al. 2013), but no reports are yet available to describe the use of these plasmids. The development of the *C. albicans* ORFeome as a Gateway® plasmid collection (Cabral et al. 2012; Chauvel et al. 2012), with a set of new *C. albicans* CIp10-based expression vectors (unpublished data) should facilitate broad applications of overexpression strategies, which have been successfully applied to the model yeast *Saccharomyces cerevisiae* (Prelich 2012).

PCR-generated cassettes are also commonly used to modify *C. albicans* genomic DNA, namely for deleting genes by iterative double crossover-mediated gene replacements (Wilson et al. 1999; Walther and Wendland 2008). They consist of a marker flanked by short regions of homology to the target sequence; these homology regions are usually 100 bp long, but can be as small as 60 bp (Wilson et al. 1999). Fusion PCR can be used to increase the size of homology regions and increase the yield of transformants (Noble and Johnson 2005). Allelic integration bias has been reported (Wilson et al. 1999), thus making deletion mutant construction a long and tedious process in some instances. Since *C. albicans* transformation is associated with genome rearrangements (Selmecki et al. 2005), particular care should also be taken in the analysis of transformants, e.g., comparing the phenotypes of several clones.

Several homologous or heterologous markers are available for selecting transformants in auxotrophic reference strains. *ARG4, HIS1,*

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LEU2, and *URA3* are the most widely used (Noble and Johnson 2007), but it should be mentioned that ectopic location of the auxotrophic marker *URA3* is linked to various effects on virulence, and should be restricted to the *RPS1* locus (Staab and Sundstrom 2003; Brand et al. 2004). In addition, dominant selectable markers could also be used in prototrophic or clinical strains. Unfortunately, the use of *S. cerevisiae* tools to study *C. albicans* is not possible due to a difference in *C. albicans* genetic code (the CTG triplet encodes a Serine instead of a Leucine). Nevertheless, a few markers have been adapted for use in *C. albicans*, namely genes providing resistance against nourseothricin and hygromycin B (Reuß et al. 2004; Shen et al. 2005; Basso et al. 2010); intrinsic resistance to geneticin (G418) makes it impossible to use the bacterial gene *KAN* as a selection marker. The *C. albicans IMH3* gene, whose overexpression allows the yeast to grow on mycophenolic acid (MPA), has also been used as a selectable marker (Köhler et al. 1997). Drawbacks of the use of this marker are that MPA-resistant clones are slow to appear, and recombination often occurs at the *IMH3* locus.

Sequential gene disruption using the same marker (either *URA3, IMH3*, or *SAT1*, encoding resistance to nourseothricin) has been made possible. The marker is flanked by direct repeats allowing its excision through homologous recombination either upon counter-selection (e.g., on 5-fluoroacetic acid for *URA3*) or induction of a site-specific recombinase (Fonzi and Irwin 1993; Wirsching et al. 2000; Reuß et al. 2004).

Several methods, very similar to those developed for *S. cerevisiae*, have been used to transform *C. albicans*. The first physical barrier encountered by the DNA is the cell wall, and the first transformation techniques developed involved spheroplasting the cells and using chemicals to help DNA pass through the plasma membrane. Although simple and quite efficient (up to 10^3 transformants/µg of transforming episomal DNA; Kurtz et al. 1987), this method requires careful attention (in monitoring cell wall digestion, avoiding cell fusion, and recovering transformants), and has been replaced by techniques involving intact cells, using either electroporation or chemicals to help DNA pass through the cell wall.

In the electroporation protocol, cells are harvested during exponential growth, and incubated with mild chaotropic agents (usually lithium acetate), rinsed and resuspended in sorbitol. They are then mixed with DNA and subjected to one or two electric pulses (Thompson et al. 1998; De Backer et al. 1999), before being resuspended in sorbitol and spread on selective medium containing 1 M sorbitol. In this protocol, adding heterologous high molecular weight DNA (so called carrier DNA) does not significantly improve transformation efficiency (Delorme 1989). Although the yield of transformants is high when compared to the chemical protocol, reaching the levels obtained with spheroplasts, the frequency of ectopic integration can be quite high (40 %, De Backer et al. 1999, and our unpublished observations).

In the chemical transformation protocol, the cells are incubated in the presence of lithium acetate, transforming DNA, polyethylene glycol (PEG) and are heat-shocked. PEG is essential to the transformation process, improving binding of DNA to the cell surface, and lithium ions act synergistically to that end (Ito et al. 1983; Zheng et al. 2005). It has also been shown that adding denatured carrier DNA and increasing the heat-shock temperature from 42 to 44 °C increased the yield of *C. albicans* transformation by ca. 100- and 10-fold, respectively (Schiestl and Gietz 1989; Gietz et al. 1995; Walther and Wendland 2003). The following mechanism has recently been proposed for chemical transformation in *S. cerevisiae*: PEG allows DNA to attach to the cell, while lithium acetate and heat shock help DNA pass through the cell wall via endocytic-like membrane invaginations (Pham et al. 2011). It has indeed been shown that some mutants defective for endocytosis exhibit a very low level of competence (Kawai et al. 2004). How DNA passes through the cytoplasm and nuclear envelope is not yet understood, although it is known that isolated nuclei can internalize DNA in an ATP-dependent process (Tsuchiya et al. 1988). For a more detailed review on mechanisms underlying yeast transformation, please see Sects. 2.1 and 2.2.

We have chosen to describe in more detail the protocol for chemical transformation, although the yield is lower than with electroporation, because it is easy to perform and does

not need any special equipment. It can also be easily scaled up and performed in 96 deep-well plates. The exact amount of DNA and resulting efficiency of transformation is highly dependent on both the genetic background of the strain and the DNA to be integrated (reviewed in Mitrikeski 2013).

7.2 Materials

1. YPD medium (1 % Yeast extract, 2 % bacto Peptone, 2 % glucose). Autoclave at 3 bar for 30 min at 110 °C.
2. 10× TE buffer: 100 mM Tris.HCl pH 7.5, 10 mM EDTA. Autoclave as previously, or filter-sterilize.
3. 10× LiAc: 1 M lithium acetate, pH 7.5, filter-sterilized. Stable for several months at 4 °C.
4. Carrier DNA (salmon- or herring-sperm DNA, usually) is used at a concentration of 10 mg/mL. Heat-denature carrier DNA at 95 °C for 10 min before first use, and then every five times.
5. 50 % (w/v) solution of PEG 3000-4000. Filter-sterilize. Store at room temperature for no longer than 1 month.
6. PEG-LiAc-TE solution is made just before use by mixing PEG to a final concentration of 40 % in 1× LiAc-TE (i.e., 8 vol. of PEG, 1 vol. of 10× TE and 1 vol. of 10× LiAc).
7. SD medium (0.67 % of yeast nitrogen base without amino acids, 2 % glucose). Autoclave as previously described.

7.3 Methods

7.3.1 Preparation of Competent Cells

1. An overnight culture is grown to saturation, at 30 °C, with mild agitation, in 5–10 mL of YPD,[1] starting from a freshly grown colony.
2. The saturated culture is diluted in 50 mL[2] of YPD to an OD600 of 0.2 (ca. 2.10^6 cells/mL), and grown at 30 °C, with gentle agitation, to mid-log phase[3] (OD600 in the range of 0.6–0.8, ca. 10^7 cells/mL).
3. Cells are centrifuged at 4 °C for 5 min at 2,000 × *g*. The pellet is washed with 10 mL of cold TE, and resuspended in 1 mL of 1× LiAc-TE solution, then transferred into a 2 mL microtube and incubated on ice for 30–60 min.

7.3.2 Transformation

1. 1–10 µg of linear DNA,[4] [5] is mixed with 5 µL of heat-denatured (single stranded) carrier DNA, in a 2 mL microtube.
2. A 50 µL aliquot of competent cells is added to the DNA, and mixed by gentle tapping on the tube.
3. 300 µL of fresh PEG-LiAc-TE solution are added, and mixed by inverting the tube.
4. The transformation mix is incubated overnight at 30 °C.[6]
5. The cells are heat-shocked in a 44 °C water bath for 15 min.[7]
6. The cells are pelleted by centrifugation for 30 s at 1,500 × *g* at room temperature and washed once with 500 µL of SD-0.4%Glc.

[1] 50 µg/mL uridine can be added to the medium throughout the procedure to increase *uri-* strains growth rate.

[2] 50 mL of culture should yield enough competent cells for up to eight transformations.

[3] The mid-log phase is usually reached in 3–4 h. URI+ cells grow faster.

[4] Transforming DNA can be a PCR-amplified cassette or a linearized plasmid.

[5] Include a negative control with no transforming DNA.

[6] Shorter incubation has been reported, but efficiency has been shown to be higher with longer incubation time. No agitation is needed.

[7] Incubation at 42 °C has been reported, but yields a lower transformation efficiency (Walther and Wendland 2003).

7. The pellet should be resuspended in 150–300 µL of SD, and the cells spread on 1–2 plates[8] of selection medium.[9]
8. Plates are incubated at 30 °C for 2–4 days.
9. Colonies are picked, and screened by PCR to check for the DNA integration.

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[8] The density of cells must not be high, since an inhibitory effect has been reported.

[9] In the case of selection of drug resistance, cells can either be plated on YPD and incubated overnight before being replica plated on the drug-containing medium, or resuspended in 5 mL of YPD, grown with gentle agitation for 3–4 h prior to plating on drug-containing plates.

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Electroporation of *Pichia pastoris*

8

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8.1 Preparing *Pichia pastoris* Cells for Transformation by Electroporation

Outlined is a protocol to harvest *P. pastoris* cells in a 25 ml culture and make them competent to take up plasmid DNA by electroporation. The protocol describes how to generate 500 µl of competent *P. pastoris* cells, enough for 15 electroporations (~30 µl of cells/cuvette). Electroporation with 200 ng of a 5,000 bp plasmid, digested for targeted integration at the AOX1 locus, can generate >50,000 transformants. More clones can be obtained using more DNA.

8.1.1 Required Reagents

1. 50 ml YPD (per liter, 10 g yeast extract, 20 g peptone and a 2 % final volume glucose, autoclave YP separately, filter sterilize the glucose).
2. 50 ml 1 M Sorbitol (filter sterilize).
3. 1 ml 1 M Hepes, pH 6.8 (sterile, autoclave).
4. 200 µl 1 M DTT (fresh solution, filter sterilize).
5. 100 ml dH_2O (sterile, autoclave)
6. 100 ml baffled shake-flask (sterile)
7. 50 ml conical tubes (sterile).
8. Refrigerated centrifuge/swinging bucket rotor for 50 ml conical tubes.
9. Tubes for freezing samples at −80 °C (microcentrifuge tubes, sterile).
10. Ice.

8.1.2 E-comp Cell Protocol

1. Inoculate YPD with a select *P. pastoris* strain; grow at 30 °C, 200 rpm. For example, inoculate 10 ml of YPD in a 50 ml conical tube, and incubate overnight. Start with healthy cells from a well-isolated colony off a fresh YPD-agar plate.
2. Prior to harvesting the culture, dilute the cells into 25 ml of YPD to a starting concentration of OD_{600} <0.2 and grow at 30 °C, 200 rpm for >5 h. Provide good aeration with a baffled 100 ml flask. Set-up the culture to harvest the cells at an OD_{600} of ~1.5.[1]

Note, *at this point put the dH_20 and 1 M Sorbitol solutions on ice so they are chilled for subsequent steps*.

3. Harvest the cells at an OD_{600} of ~1.5. Do not let the culture grow past an OD_{600} of 1.7. Initially, place the culture on ice for 15 min. Then, centrifuge the cooled cells at 4 °C, 2,000 rpm for 5 min (RCF 750 *g*). Save the yeast pellet and discard the supernatant.[2]

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4. Re-suspend the cell pellet in 2 ml YPD-Hepes (1.6 ml YPD + 0.4 ml 1 M Hepes pH 6.8). Slowly add 75 µl freshly made 1 M DTT to the YPD/Hepes/cell suspension and rock cells at ~100 rpm for 25 min at 25–30 °C.
5. Dilute the sample containing DTT by adding multiple volumes of sterile, ice-cold dH_2O. For example, add 40+ ml ice-cold dH_2O to the sample in a 50 ml conical tube. Keep cells at 0–4 °C from here on out.
6. Pellet the cells by centrifugation at 2,200 rpm (RCF ~800 *g*) for 5 min in a refrigerated centrifuge at 4 °C, and discard supernatant. All subsequent centrifugation steps will be at this temperature/speed/duration.
7. Wash cells a second time in a similar volume of ice-cold dH_2O. Completely re-suspend the cell pellet by vortexing the sample, centrifuge and discard the supernatant.
8. Wash cells in sterile, ice-cold 1 M Sorbitol. Re-suspend cells in 20 ml 1 M Sorbitol, then centrifuge the cells as above and discard the supernatant.[3]
9. Repeat the 1 M Sorbitol wash, as above; however, this time remove as much of the liquid from the cell pellet as possible.
10. Add 300 µl of ice-cold 1 M Sorbitol to the final pellet and re-suspend the cells completely (work the cells into solution by pipetting and vortexing). Keep the final cell suspension on ice.
11. These E-comp cells can be used immediately, or frozen in chilled tubes for storage at −80 °C. Add ~30 µl of E-comp cells to an ice-cold, sterile, micro-centrifuge tube for "one-shot" applications, and place the tubes in the −80 °C freezer. E-comp *P. pastoris* cells can be stored −80 °C for ~6 months without large losses in competency.

8.2 Electroporation of Plasmid DNA into E-Comp *P. pastoris* Cells

Plasmid expression vectors are routinely electroporated into *P. pastoris* for integration into the genome and expression of heterologous proteins (Becker and Guarente 1991; Cregg et al. 2009). Electroporation is effective for plasmids with either dominant selectable marker genes, such as those for drug resistance (e.g., Nourseothricin (Nat), G418 and Zeocin), or biosynthetic genes to complement mutations in the transformed cells (i.e., the HIS4 or ADE2 gene; Cregg et al. 1985; Lin-Cereghino and Lin-Cereghino 2007).

Note, *the following protocol uses cuvettes with a 1 mm gap-width for electroporation; most published protocols for P. pastoris use 2 mm cuvettes. Furthermore, be aware that pre-programmed electroporation settings may be set for 2 mm cuvettes. The protocols for either type of cuvette are acceptable; however, the appropriate electroporation settings must match the cuvette gap-width. Higher voltages required for the 2 mm cuvettes will cause arching with the 1 mm cuvettes.*

BioGrammatics prefers a 1 mm cuvette with a v-shaped bottom and capacity for ~100 µl (e.g., USA Scientific catalog #9104-1050).

8.2.1 Plasmid DNA

Expression vectors are most often electroporated into *P. pastoris* cells as linear DNA molecules (Cregg 2007; Lin-Cereghino and Lin-Cereghino 2007). The DNA "ends" facilitate integration into the *P. pastoris* genome to create stable expression strains. Circular plasmid DNA, linearized within a region of homology to a genomic target-locus, is more likely to integrate at the targeted site and results in higher numbers of transformants than when DNA without homology integrates at "random" sites. For example, the restriction enzyme *Pme*I recognizes the 5′-GTTTAAAC-3′ site in the middle of the AOX1 promoter (pAOX1). Vectors linearized at the *Pme*I site in the pAOX1 preferentially integrate into the AOX1 promoter in the *P. pastoris* genome by a single "cross-over" event that results in a duplication of the pAOX1 (Fig. 8.1).

The linear DNA should be cleaned and concentrated after digestion, prior to transformation. DNA in water, rather than a solution with salts is better for electroporation to reduce the conductivity of the sample during the electro-

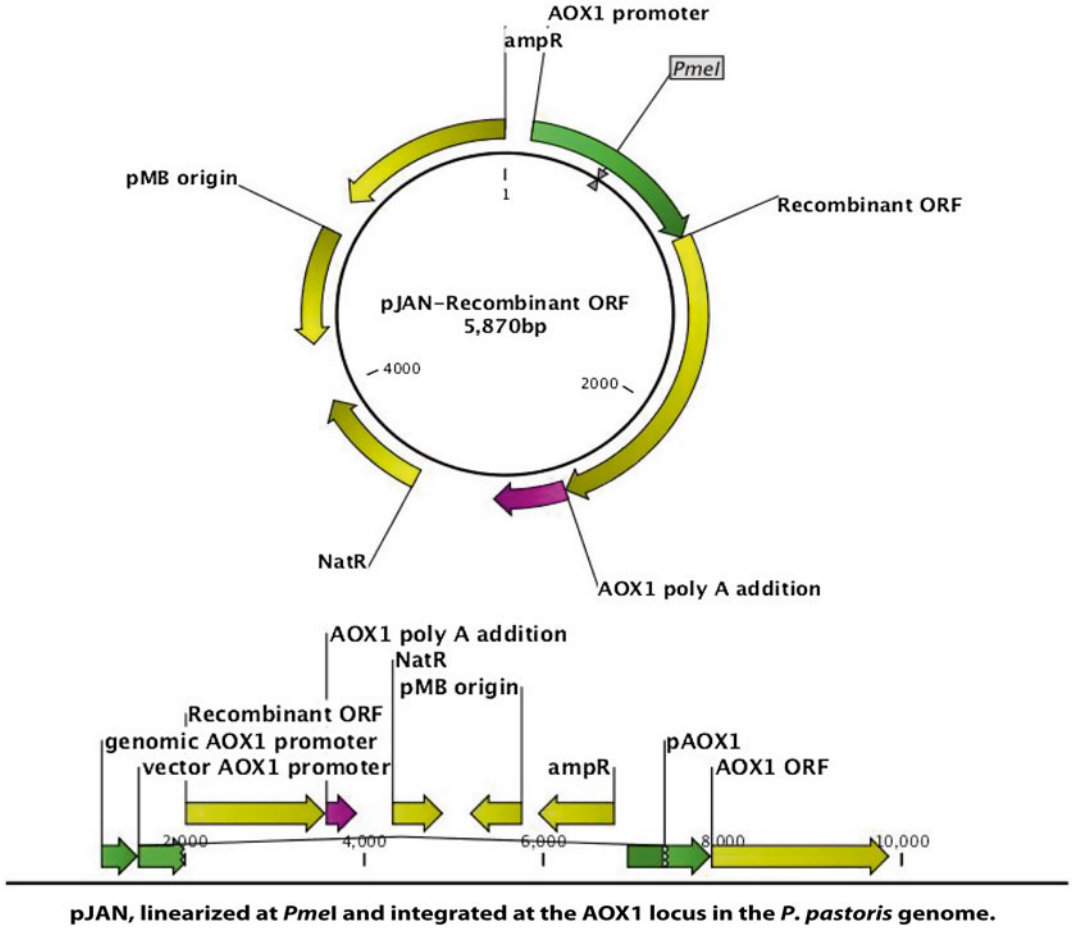

Fig. 8.1 Model of the predicted insertion of a *P. pastoris* expression vector at the AOX1 locus in the *P. pastoris* genome

poration. DNA suspended in 10 mM Tris, 1 mM EDTA (TE) can be used; however, the volume of the DNA-TE sample added to the E-comp cells must be limited to< 20 % of the E-comp cell volume.

8.2.2 Required Reagents

1. E-comp cells.
2. DNA (prepared for electroporation).
3. Electroporation cuvettes (sterile).
4. Electroporation apparatus.
5. *Pichia* Electroporation Recovery Solution (PERS, YPD:1 M Sorbitol, 1:1 v/v, sterile).
6. Ice.

8.2.3 Electroporation Protocol

1. Label, then chill, sterile 1 mm electroporation cuvettes on ice at least 5 min prior to electroporation.
2. Remove E-comp cells from −80 °C freezer. Thaw and place on ice.[4]
3. Add DNA to cells. Results with DNA volumes up to 5 µl per 30 µl of E-comp cells are similar; larger volumes may result in lower numbers of transformants. Most importantly, reduce the amount of salt/ions added to the E-comp cells with the DNA to minimize conductivity of the sample during electroporation. DNA in water or low concentrations of Tris is best.[5]

Table 8.1 Electroporation settings

Instrument	Cuvette gap (mm)	Sample volume (µl)	Charge voltage (V)	Cap. (µF)	Resistance (Ω)	Expected Pulse length (~m)
ECM 399, or 630 (BTX); BioRad Gene Pulser I.	1	25–30	1,150	25	200	5
Eppendorf, Eporator®, Multiporator®.	1	25–30	1,200	No setting	No setting	5
BioRad (Gene Pluser Xcell®, MXcell®, II, and *E. coli* Pulser®)	1	25–30	1,150	10	600	5

4. Gently mix the DNA and E-comp cells and transfer the entire sample to a cuvette. Make sure the sample is inserted between the metal plates and keep the cuvette/sample on ice.
5. Rapidly, remove the sample cuvette from the ice and place it between the electrodes in the "shock chamber" of your electroporation device; activate and discharge the device (all in ~5 s; Table 8.1).[6]
6. After the electroporation discharge, add ~1 ml *Pichia* Electroporation Recovery Solution (PERS) to the cuvette, mix it with the cells and then transfer the sample from the cuvette to a sterile 1.5–2 ml micro-centrifuge tube for incubation.[7]
7. Incubate samples at 30 °C, shaking at ~100 rpm for approximately 3–4 h. Shorter recovery times will yield fewer transformants; longer times can result in cell division and "sister" clones.
8. Spread the cells onto YPD-agar plates with the appropriate drug, or to minimal plates for auxotrophic selection, and incubate the plates at 30 °C for 2 days. For example, plate 100 µl of the sample on one plate, and the remainder on second plate. Centrifugation at 8,000 rpm in a microfuge for 30 s will pellet cells from PERS for plating. Drug concentrations for selection include: G418 at 750–1,000 µg/ml, and Nourseothricin (Nat), or Zeocin, at 100 µg/ml.
9. Carefully pick cells from a single colony on an original selection plate and streak them out on a second selection plate so single colonies can again be isolated. Incubate at 30 °C overnight. All subsequent testing should be performed with cells originating from this 2nd selective plate. Furthermore, no selection is required for stably transformed cells in subsequent testing.
10. Glycerol stocks of select clones should be made with cells from the same single colony from the 2nd selective plate as were used for the testing. Add sterile glycerol to an overnight YPD culture, (30 % v/v glycerol).

8.3 Notes

1. Wild type *Pichia* can double in ~2 h. Note, the yeast culture must be diluted to an OD_{600} of< 0.3 to accurately measure the absorbance.
2. *P. pastoris* cells sediment at a RCF of 750 *g*; smaller cells, like contaminating bacteria, will remain in suspension and/or pellet as a layer above the yeast. Start over if any contamination is observed.
3. Centrifuge cells with a swinging bucket rotor, recovering cells from 1 M Sorbitol is more difficult with a fixed angle rotor.
4. Hand warming tubes of frozen E-comp cells from −80 °C storage during transported from the freezer works well (rapid thawing may be slightly better than a slower thaw).
5. Electroporation with 200 ng of a linear expression vector can generate thousands of transformants, if integration is targeted at the AOX1 locus; efficiencies at other loci vary. More DNA will result in more transformants, up to ~1 µg of DNA/sample.
6. Warming of the cuvette/sample prior to electroporation can significantly reduce the number of transformants. The time after the sample cuvette is removed from the ice bath and placed in the Electroporator shock chamber, until the current is discharged, is critical.

This "ice-to-Zap" time should be as short as possible. In general, electroporation with 1 mm cuvettes should be conducted at a voltage of 1,150 V (1,200 V if 1,150 V is not possible), with 25 uF and 200 Ω, or 10 uF and 600 Ω. The actual time constant depends on the total conductivity of the sample and is best between 4 and 6 milliseconds.

7. The original tube provided with the E-comp cells can be used to incubate cells after electroporation. Additionally, inverting the electroporation cuvette to draw the sample out from between the electrodes with a pipet tip helps to extract more of the sample for recovery and plating.

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Insertional Mutagenesis of the Flavinogenic Yeast *Candida famata (Candida flareri)*

9

Kostyantyn Dmytruk and Andriy Sibirny

9.1 Introduction

Insertional mutagenesis is the method for simplified identification and cloning of a target gene. The principle of the method is based on the generation of mutations by integration of a nucleotide sequence, so-called insertion cassette, to the genome of the host cell resulting in alteration or limitation of target gene expression. The mutated gene is tagged by the insertion cassette. Identification of the mutated gene is performed via isolation of the insertion cassette with flanking genomic DNA sequences. There are several types of insertional mutagenesis like transposon mutagenesis, retroviral insertional mutagenesis and Restriction Enzyme-Mediated Integration (REMI) mutagenesis differing mostly by site-specific or nonspecific integration mechanisms (Garfinkel and Strathern 1991, Uren et al. 2005, Dmitruk and Sibirnyi 2007).

There are two main prerequisites for efficient gene tagging: first, an adequate transformation frequency; second, random integration of the insertion cassette and a high percentage of single-copy integrations into the genome of the recipient cells.

Insertional transformants with desired phenotypes are screened on selective media. Transformant selection requires selective markers providing sufficient transformation frequency. Therefore, a selective marker is an obligatory element of the insertion cassette.

Dominant markers have an advantage, because, unlike auxotrophic markers, they do not need preliminary selection of the recipient strain. Additionally, dominant markers are advantageous for systems with high homologous recombination frequencies, since dominant markers are typically heterologous and their sequences do not exhibit homology with the recipient genome enabling random integration (Lu et al. 1994).

After selection of mutants with the desired phenotype, the number and complexity of insertion events are examined by Southern blot analysis using part of the insertion cassette as a probe.

The insertion cassette might have a bacterial origin of replication (ori) and a selective marker for selection in *Escherichia coli* if the identification of the integration site will be performed through *E. coli*. In this case, the insertion cassette is isolated from the genomic DNA together with the flanking regions via digestion of the total DNA of the selected strain by restriction enzymes that do not cut in the insertion cassette, subsequent self-ligation and transformed to *E. coli.* Plasmids harboring insertion cassettes with chromosomal

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flanking regions isolated from *E. coli* are subjected to sequencing using primers complementary to the vector backbone (Kahmann and Basse 1999). Alternatively, the integration site could by identified by PCR. Synthetic linkers are ligated to the ends of the chromosome DNA fragments released after corresponding restriction endonuclease treatment. Primers homologous to the insertion cassette, and linkers, are used for PCR amplification of the cassette flanking regions. Resulting PCR-products are sequenced (Kwak et al. 1999). In the latter case, elements providing plasmid replication and selection in *E. coli* are not obligatory.

Finally, it is essential to prove that the observed phenotype is caused by the integration of the insertion cassette at the particular site. Two main approaches are used most often. First, complementation of the mutation by introducing the corresponding wild-type gene. Second, the insertion cassette with flanking regions is used for integration into the genome of a wild-type strain via homologous recombination. The resulting strain is compared to the original insertion mutant by Southern and phenotypic analysis. The first approach is faster and could be used in organisms with inefficient homologous recombination (Dmitruk and Sibirnyi 2007).

In this chapter, an optimized procedure for mutant isolation with subsequent gene tagging via insertional mutagenesis for the flavinogenic, salt-tolerant yeast *Candida famata (Candida flareri)* is described. A highly efficient protocol for electrotransformation of *C. famata* as well as selective markers and corresponding selective media are provided.

9.2 Materials

9.2.1 Reagents

1. Double distilled water (dd-water), or equivalent
2. Peptone
3. Yeast extract
4. Sucrose
5. Glucose
6. Agar
7. Yeast Nitrogen Base w/o A.A. (DIFCO)
8. 6 N HCl
9. 6 N KOH
10. Mycophenolic acid
11. p-Fluoro-DL-phenilalanine
12. L-Tyrosine
13. Phleomycin (InvivoGen)
14. 1,4-Dithiothreitol (DTT)
15. K_2HPO_4.
16. KH_2PO_4
17. NucleoSpin Tissue Kit (Macherey-Nagel)
18. DNeasy Blood & Tissue Kit (Qiagen)
19. Wizard® *Plus* SV Minipreps DNA Purification System (Promega)
20. Restriction enzymes (Fermentas)
21. T4 DNA ligase (Fermentas)

9.2.2 Equipment

1. 1.5-mL microcentrifuge tubes
2. 50-mL centrifuge tubes
3. Graduated cylinders and beakers
4. Lockable storage bottles
5. Plastic petri dishes
6. Vortex, e.g. Fisher Scientific
7. Filters for cold sterilization
8. Micropipettors, e.g. Eppendorf
9. Micropipettor tips
10. Equipment for agarose gel electrophoresis, e.g. BioRad
11. Shaker for 28 °C
12. Microcentrifuge, e.g. Eppendorf 5417R
13. Centrifuge, Eppendorf 5804R
14. Incubator on 28 °C
15. pH meter
16. Analytical balance (0.1 mg readability)
17. Laboratory balance (capacity 100 g)
18. Electroporator ECM 600 produced by BTX
19. 2-mm electroporation cuvette

9.3 Methods

9.3.1 Selective Media and Selective Markers

Yeast cells are cultured on YPD media (0.5 % yeast extract, 1 % peptone, and 2 % glucose) or synthetic defined (SD: 0.67 %, yeast nitrogen

Table 9.1 Selective markers for transformation of *C. famata*, concentration of the selective agents and average transformation frequencies

Gene	Source	Transformation frequency (transformants/ µg of DNA)	Medium	Selective agent	Concentration (mg/L)	Source
LEU2	*S. cerevisiae*	200	SD	Leucine lacking	—	Dmytruk et al. (2006)
ble	*S. aureus*	100	YPD	Phleomycin	2–4	Dmytruk et al. (2006)
IMH3	*D. hansenii*	30	SD	Mycophenolic acid	15–20	Dmytruk et al. (2011)
ARO4	*D. hansenii*	100	SD	p-fluorophenylalanine Tyrosine	2,500 800	Dmytruk et al. (2011)

base without amino acids, DIFCO, and 2 % glucose). Agar is (1.5 %) added to solidify media. Adjust pH to 6.0 while stirring, with 6 N KOH, if required. For selection of yeast transformants on YPD, 3 mg/L phleomycin is added. For selection of yeast transformants on SD, 15 mg/L mycophenolic acid or 2.5 g/L fluorophenilalanine together with 0.8 g/L tyrosine are added. Transformants of the auxotrophic *C. famata* L20105 (*leu2*) strain could be selected on SD lacking supplements using an insertion cassette harboring the *LEU2* gene of *Saccharomyces cerevisiae* coding for β-isopropylmalat dehydrogenase (Voronovsky et al. 2002). Phleomycin resistant transformants can be selected after transformation of *C. famata* with a plasmid harboring the *ble* gene from *Staphylococcus aureus* under control of the strong constitutive promoter of the homologous *TEF1* gene encoding the translation elongation factor 1 (Dmytruk et al. 2006). The *Debaryomyces hansenii IMH3* gene coding for inosine monophosphate dehydrogenase, serves as a dominant selective marker conferring resistance to mycophenolic acid (Dmytruk et al. 2011). The modified version of the *D. hansenii ARO4* gene (coding for 3-deoxy-d-arabino-heptulosonate-7-phosphate [DAHP] synthase), which catalyzes the first step in aromatic amino acid biosynthesis and is insensitive to feedback inhibition by tyrosine, driven by the *CfTEF1* promoter can be used as dominant selective marker conferring resistance to fluorophenylalanine (Dmytruk et al. 2011).

Average transformation frequencies of *C. famata* with above mentioned selective markers and corresponding selective media are summarized in Table 9.1.

9.3.2 Electrotransformation of Yeast *C. famata*

This method has been used to select integrants of *C. famata* with transformation frequencies of 30–200 transformants per µg of linearized plasmid DNA depending on the type of selective marker and selective media.

1. Inoculate fresh cells in 3 mL YPD and cultivate at 28 °C while shaking at 200 rpm for approximately 24 h.
2. Add 5–20 µL of the obtained culture to 100 mL of YPD medium in a 300 mL flask and cultivate overnight at 28 °C to an OD_{600} of 1.5–2.0. The growth phase of the culture is one of the critical parameters.
3. Harvest the cells (3,500 rpm for 10 min), suspend in 40 mL of 50 mM Phosphate buffer, pH 7.5, containing 25 mM DTT (To prepare 100 ml of 10× stock solution of Phosphate buffer mix 85 ml of 0.5M K_2HPO_4 and 15 ml of 0.5M KH_2PO_4. Adjust pH to 7.5 under stirring, with 6 N KOH. Mix 0.1544 g of DTT with 4 mL of stock solution. Add dd-water to a final volume of 40 mL and filter sterilize.) and incubate for 15 min at 28 °C.
4. Spin down cells and wash three times with 100 mL of cold water at 4 °C.
5. Pellet the cells and resuspend them in 40 mL of cold 1 M sucrose.
6. Harvest the cells (3,500 rpm for 10 min at 4 °C), suspend in 1.2 mL of cold 1 M sucrose.
7. Add 10 µg of linearized insertion cassette to 200 µL of cell suspension (approximately 2×10^8 cells).

8. Tap the mixture on the bottom of a pre-chilled 2-mm electroporation cuvette.
9. Carry out the electroporation. Use the following electroporation conditions: electroporator ECM 600 (BTX); field strength 11.5 kV sm^{-1}; capacitance 50 μF; resistance 129 Ω (R5) resulting in pulse length around of 4.5 ms.
10. After electroporation, quickly add 1 ml of 1 M sucrose to the cuvette.
11. Transfer 1 mL of the transformed cell suspension to a 15 mL glass tube, add 1 mL of 2×YP (1 % Yeast extract, 2 % peptone) and cultivate at 28 °C with shaking 200 rpm for approximately 1–4 h.
12. Spread the suspension of transformed cells onto selective YPD plates and incubate at 28 °C for 3–5 days.
13. In case of using selective SD medium, first harvest the transformed cells and wash them twice with sterile water, before plating.

9.3.3 Isolation of the Insertion Cassette Together with Flanking Regions

This method has been used to isolate the insertion cassette together with flanking regions from yeasts in general. Modifications may be needed to extract DNA from different yeast species. The method describes isolation of the insertion cassette harboring elements providing plasmid replication and selection in *E. coli.*

1. Isolate genomic DNA from selected and characterized, by Southern blot analysis, transformants using protocol for yeast NucleoSpin Tissue Kit (Macherey-Nagel).
2. Determine quality and quantity of prepared samples of chromosomal DNA by agarose gel electrophoresis.
3. Digest 5 μg of chromosomal DNA with appropriate restriction enzymes that do not cleave the cassette, in 50 μL total volume.
4. Purify digested DNA with the DNeasy Blood & Tissue Kit (Qiagen).
5. Self-ligate digested and purified DNA with 10U of T4 DNA ligase in a total volume of 50 μL for overnight at room temperature.
6. Purify self-ligated DNA with the DNeasy Blood & Tissue Kit (Qiagen).
7. Transform 0.2 volume of the purified ligation mixture into electrocompetent *E. coli* .
8. Isolate plasmid DNA from *E. coli* with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA).
9. Perform the sequencing of isolated plasmids for identification of the insertion locus with primers homologous to sequence of insertion cassette.

9.4 Special Precautions

1. Ensure that cells were harvested at appropriate growth phase.
2. Ensure that all traces of media and buffers are removed completely since they affect subsequent steps in the transformation procedure.
3. Ensure that the solution of 1 M sucrose is added and the sample is mixed immediately after electroporation.
4. Ensure that most of the steps (including centrifugation) are done at 4 °C

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Part IV

Transformation Methods: Particle Bombardment

Biolistic Transformation for Delivering DNA into the Mitochondria

10

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10.1 Introduction

The Biolistic procedure is a method of genetic transformation that uses helium pressure to deliver particles and introduces DNA-coated microcarriers into cells. The technique has become a widely utilized system for the transformation of a wide range of organisms including insects (Yuen et al. 2008), plants (Becker et al. 2000; Fernando et al. 2000; Fukuoka et al. 1998; Klein et al. 1988; Maenpaa et al. 1999; Rasco-Gaunt et al. 1999; Tang et al. 1999), animals such as *Caenorhabditis elegans* (Isik and Berezikov 2013), animal cells (Johnston 1990), algae (Mayfield and Kindle 1990), bacteria (Shark et al. 1991), fungi (Bills et al. 1995; Durand et al. 1997; Fungaro et al. 1995; St. Leger et al. 1995), and intracellular organelles (Bonnefoy et al. 2007; Larosa and Remacle 2013).

Both stable and transient transformation is possible with the biolistic particle delivery system. Moreover, the particle delivery is a convenient method for transforming intact cells in culture since there is no need for pre- (competent cells) or postbombardment (transformation recovery) manipulation even if the biolistic transformation, compared to other procedure, is "inefficient" in the sense that most cells are killed by the microprojectile bombardment. In general, the selection of survivors and the identification of the appropriate recombinants are based on a positive selection, supported by selection markers. It is widely recognized that transformation efficiency decreases remarkably under nonselective conditions. For example, the transformation efficiency of potato under nonselective conditions was only about 1/400 of that under selective conditions (Kaya et al. 1990).

For fungi, the biolistic approach is particularly effective when protoplasts are difficult to obtain and/or the organisms are difficult to culture. This is particularly applicable to *Arbuscular mycorrhizal* fungi, as they are obligate symbionts that can only be propagated in association with intact plants or root explants. Furthermore, these fungi are aseptate and protoplasts cannot be released (Harrier and Millam 2001).

Two genes were introduced in *Cercospora caricis* by biolistic transformation: the b-glucuronidase gene (GUS) fused to the *GDP1* promoter of *Cochliobolus heterostrophus* and the hygromycin B resistance gene under control of the *ptrpC* promoter of *Aspergillus nidulans*. Although the transformation frequency was not high, all transformants were stable when they

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were propagated on a selective medium after eight subsequent transfers (Aly et al. 2001).

Among the developed transformation systems for the pathogenic yeast *Candida parapsilosis*, the biolistic procedure was not very efficient and resulted in about 5×10^2 transformants/μg of plasmid DNA, whereas the chemical method using LiCl or $CaCl_2$ yielded about 1×10^3 transformants/μg of DNA. The electroporation procedure was an order of magnitude more efficient than the chemical method (Zemanova et al. 2004).

Yu and Cole (1998) report the first stable integration of plasmid DNA into chromosomes of *Coccidioides immitis*, which is a respiratory fungal pathogen of humans, by the biolistic DNA delivery method.

Genetic transformation of the ectomycorrhizal fungus *Pisolithus tinctorius* has been also successfully performed by microprojectile bombardment and *Agrobacterium*-mediated transformation. This last method proved to be the more efficient. The visualization of GFP-associated fluorescence in saprophytic mycelia confirmed the expression of the reporter gene (Rodriguez-Tovara et al. 2005).

Te'o et al. (2002) have successfully defined a protocol for the biolistic transformation of intact conidia from the filamentous fungus *Trichoderma reesei* using the more advanced Hepta Adaptor assembly (Bio-Rad Laboratories). This machine offers potential for further increasing the efficiency of biolistic transformation by particle delivery from seven barrels to one plate.

In yeast, different procedures have been developed to transform intact cells (Mitrikeski 2013). Chemical and physical approaches have been described, including the biolistic procedure (Armaleo et al. 1990) leading to transformation frequencies between 10^{-5} and 10^{-4} which is quite low compared to the frequency obtained with other transformation protocols (between 10^3 and $>10^6$). However, this method is the only way to transform cytoplasmic organelles such as mitochondria (Johnston et al. 1988; Fox et al. 1988).

10.2 The Biolistic Transformation

10.2.1 The Biolistic Transformation Process

The Biolistic PDS-1000/He device (Kikkert 1993) used for transforming cells is shown in Fig. 10.1. The helium pressure released by a rupture disk, and vacuum in the PDS-100/He system accelerates a plastic sheet (macrocarrier) loaded with millions of microscopic tungsten or gold particles (microcarriers) coated with recombinant DNA into target cells at high velocity. The macrocarrier is halted after a short distance by a metal grid (stopping screen); see Fig. 10.2. The DNA-coated microcarriers penetrate and transform the cells.

The launch velocity of microcarriers for each bombardment is dependent upon the helium pressure (rupture disk selection), the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the macrocarrier, the macrocarrier travel distance to the stopping screen, and the distance between the stopping screen and target cells (Figs. 10.1 and 10.2). The particles can be coated with different substrates, from purified plasmid in most cases to bacteriophage lambda, yeast, and bacterial cells as projectiles to deliver marker/reporter genes into organisms (Rasmussen et al. 1994; Kikkert et al. 1999). After the target tissue to be transformed is placed into the chamber, the door is closed and a vacuum is applied. Activating the fire switch allows helium to flow into the gas acceleration tube at a rate regulated by the helium metering valve and monitored by the helium pressure gauge. The gas is held until the burst pressure (usually from 450 to 2,200 psi) of the rupture disk is reached. This generates a helium shock wave into the bombardment chamber. The shock wave is generated by rupture of a membrane by the high pressure and accelerates a second membrane (macrocarrier) holding DNA-coated microprojectile particles toward the plate. A stopping screen placed between the macrocarrier assembly and the tissue retains the plastic sheet, while

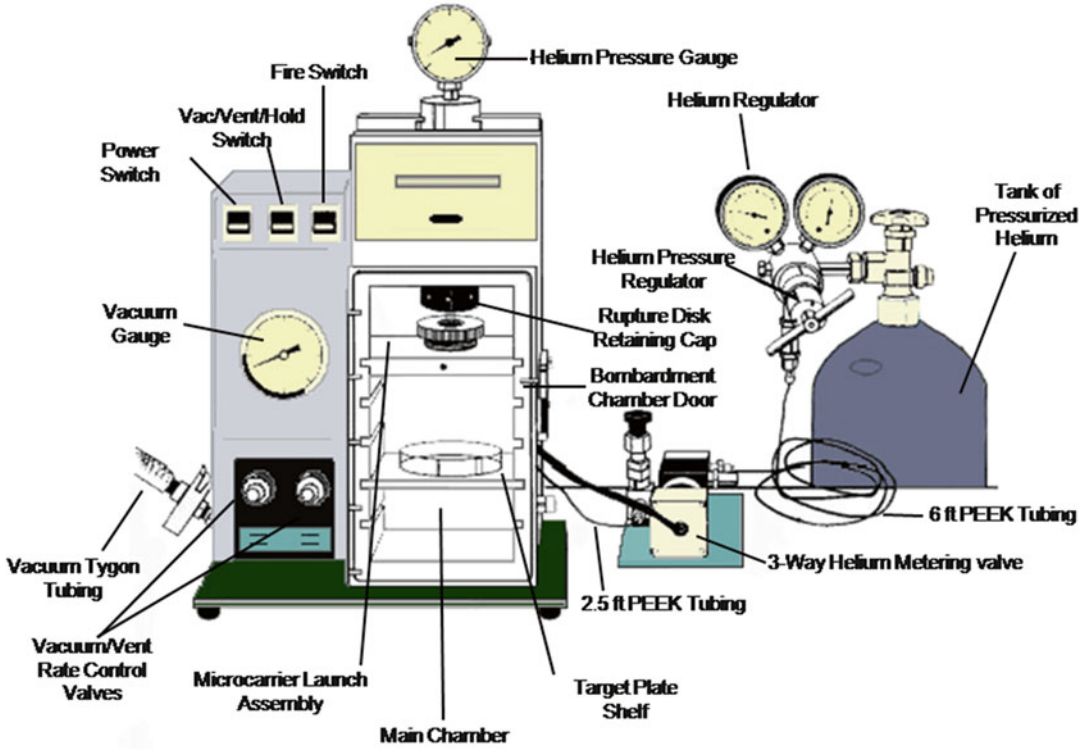

Fig 10.1 Schematic diagram of the PDS-1000/Helium particle bombardment device

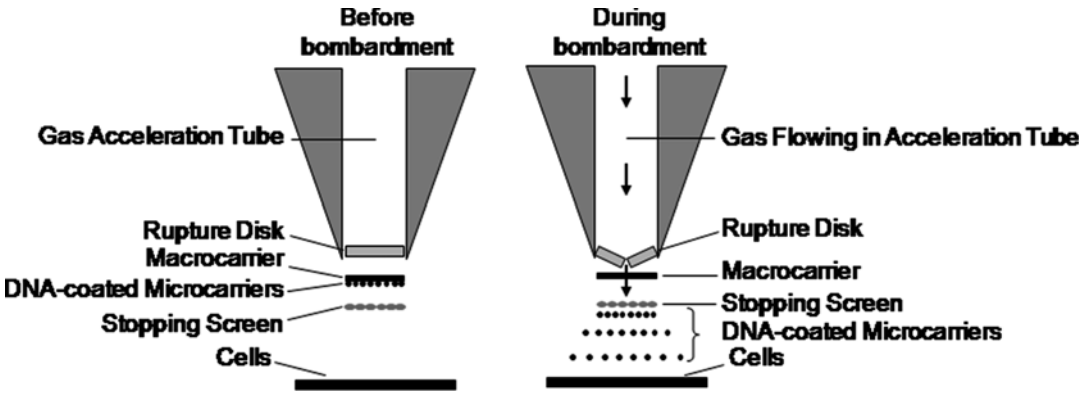

Fig 10.2 Schemes of the effect of the shot on the microcarries launch assembly

allowing the particles to pass through at high velocity and transform the cells/tissue, resulting in transient and/or stable transformation. In addition, the mitochondria of a small fraction of such transformants also take up the DNA.

Factors affecting bombardment efficiency are numerous, and interact in complex ways (Sanford et al. 1993). Biological parameters (cell types, growth condition, cell density, and osmoticum condition) and setting instruments (particles type and size, vacuum and pression levels, and target distance) are important variables.

There are several advantages in using gold particles as they tend to be very uniform, thus

allowing for optimization of size relative to cell type. Moreover, gold is not toxic to the cells and does not catalytically attack the DNA bound to it, as observed for tungsten particles. The main disadvantage to utilize gold particles is that they are expensive and not stable in sterile aqueous suspensions. Over a period of time gold agglomerates irreversibly and therefore it is best to prepare gold particles on the day of use. The coating of the gold particles is one of the key points of variation affecting biolistic efficiency. The gold particles and plasmid DNA must be prepared rapidly (mixing plasmid DNA, spermidine, and calcium chloride).

Moreover for successful biolistic transformation, the target cells/tissue must be receptive to transformation, have potentially high rates of particle penetration, and obviously maintain cell survival and growth capability. If possible spores are the preferable fungal target tissue for biolistic transformation studies; they are particularly amenable to transformation studies as they can be surface sterilized, are available in large quantities, and can be easily checked for damage and subsequent growth and development postbombardment. For example, the choice between cells or spores for the transformation of *Gigaspora rosea* was a significant factor since this species has relatively large spores (230–305 μm diameter) and a thin spore wall ranging from 2.4 to 7.5 μm in thickness. The latter, would allow easier penetration of the microprojectile particles hence a better transformation efficiency (Harrier and Millam 2001).

10.2.2 Plasmid and Selection in Nuclear Transformation

One of the most critical factors to be considered in biolistic transformation is the choice of vectors to be utilized. Vectors containing transposable elements may greatly increase the efficiency of integration into the genome following biolistic transformation (Laufs et al. 1990).

To enable monitoring and selection of transformed material, the plasmid constructs must have appropriate reporter or selective genes with suitable promoters and may either be replicating or integrative. The size and form of the transforming DNA should also be considered. DNA can be introduced in many forms including: circular, linear, single-stranded, and/or double-stranded DNA.

The choice of the plasmid is based on the way in which they are maintained after transformation: integrating vectors, where the plasmid DNA is integrated into the nuclear genome by recombination event, or autonomously replicating vectors. All the vectors however have a selectable marker that, under selective conditions, allows only for growth of the transformed cells among a population of cells mutated for that marker gene. Usual selection systems are based on antibiotic selectable markers, the most common being Tet and G418, or common auxotrophic markers.

The anaerobic fungus *Neocallimastix frontalis* has been biolistically transformed using plasmids containing the bacterial beta-glucuronidase gene (GUS) fused to the promoter sequences of the enolase gene from *N. frontalis*. Transformants were detected by histochemical assay for beta-glucuronidase (Durand et al. 1997).

Similar plasmid was used for transformation of *G. rosea* and transient GUS gene expression was detected in 40–50 % of spores by colorimetric and immunological based methodologies.

This construct bears a *GUS* gene, assembled by ligating the *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpd*) promoter to the coding sequence of the *Escherichia coli gusA* (formally *uidA*) gene (Roberts et al. 1989); two analytical strategies were employed, the immunodetection of the GUS protein and colorimetric detection for selection of transformants.

10.3 The Mitochondrial Transformation

Nucleic acids don't generally penetrate into mitochondria. Although there are some specific import of small RNA and tRNA molecules (Mahapatra and Adhya 1996; Tarassov et al. 2007; Sieber et al. 2011) they do not allow any kind of DNA into mitochondria; consequently it was impossible for

many years to manipulate the mitochondrial genome as was done for the nuclear one. Nowadays, some systems have been developed based on tRNA import (Tarassov et al. 2007; Salinas et al. 2008) but they require sophisticated tools and do not allow for any mutation to be introduced into the mitDNA. While the biolistic transformation is not very helpful for the *Saccharomyces cerevisiae* nuclear DNA, for which more efficient alternative transformation methods (spheroplasts, cells treated with lithium salts, and electroporation) exist, it has however been a major breakthrough for mitochondrial reverse genetics.

The biolistic procedure was initially described by Johnston et al. (1988) and Fox et al. (1988) but has now been developed into a routine procedure (Bonnefoy and Fox 2007). Since then, it has permitted to construct specific mitochondrial-encoded mutations or to introduce specific genes to the mitochondria (Rinaldi et al. 2010).

Because of its properties, *S. cerevisiae* is a particularly well-studied simple organism; it has been widely used to unravel basic cellular functions (basic mechanisms of replication, recombination, cell division and metabolism are generally well conserved between yeast and higher eukaryotes, including mammals). It also offers invaluable guidance for approaching human disease-associated gene functions. Furthermore, the ease of genetic manipulation of yeast allows its use for conveniently analyzing and functionally dissecting gene products from other eukaryotes.

The importance of yeast is especially true for mitochondrial studies since (1) yeast cells can survive without functional mitochondria. Any strain deficient for mitochondrial function will not grow on respiratory substrates such as glycerol or ethanol containing media but can be cultivated and analyzed on fermentescible media such as glucose; (2) a large collection of mitochondrial mutants is available; (3) an active homologous recombination system exists (Bolotin et al. 1971) which can be used to reassociate alleles, and (4) bacterial plasmids (carrying or not mitDNA genetic information) can be replicated in the yeast mitochondria; yeast bearing a "mitochondrial plasmid" are called synthetic *rho*$^{-}$ (Fox et al. 1988).

It is therefore not surprising that with the help of the biolistic transformation, many new mutations were created since the first publications of Meunier (2001) and Feuermann et al. (2003), mostly with the goal to study mitochondrial pathological mutations. Yeast mutants bearing mitochondrial substitutions equivalent to human mitochondrial mutations, created by biolistic transformation, can indeed allow to distinguish between a great number of neutral mitochondrial substitutions and pathogenic mutations, a problem that in human is difficult to solve because of the high mutational rate of the mitochondrial genome and the presence of polymorphisms (Tuppen et al. 2010). Moreover in yeast it is possible to screen for compensatory mutations or to isolate suppressing genes that rescue the defective phenotype of the original mutation. In addition, it is easy to change the nuclear background for the same mitochondrial genome (see Sec. 10.3) and analyze the effect of a given mitochondrial genotype with different nuclear ones. Finally, the fact that yeast becomes very rapidly homoplasmic when cells with two mitochondrial populations are crossed, leads to the simplification of a very complex system as is the human one.

The mitochondrial transformation is not an efficient procedure; more than 99 % of the cells are killed by the process and among the survivors, about only 1 of 500 or 1,000 are mitochondrial transformants (Rohou et al. 2001). Nevertheless this inefficiency of transformation can be overcome with a strong positive selection.

10.3.1 The Parameters

10.3.1.1 The Choice of Recipient Strains

Strain background is an important factor affecting the efficiency of mitochondrial transformation (Bonnefoy and Fox 2007). Excellent hosts for mitochondrial transformation, derived from DBY947 (S288C background), are the MCC109 (MATα, ade2-101, ura3-52, karl-1) and its isogenic MATa MCC123 strains (Costanzo and Fox 1993). Other strains such as W303 derivatives can also be used, but any new strain has to be

checked in reference to the previous ones. One can also wonder what strain (*rho*$^+$ or *rho*°) should be used. Let's recall that *rho*$^+$ strain contains a complete mitochondrial genome while *rho*$^-$ or *rho*° have partial or complete deletions of the mitochondrial genome, respectively. It has been reported that mitochondrial transformation is 10–20 times more efficient bombarding a *rho*° strain than an isogenic mitochondrial mutant *rho*$^+$ strain; nevertheless the use of a *rho*° background requires an additional step and is therefore a lengthier process. Consequently, the *rho*$^+$ background has only been used if a direct positive selection can be applied (i.e. selection of antibiotic resistant mutant, selection of respiratory competent cells among respiration deficient one, or selection of arginine prototroph among arg$^-$ cells using a mitochondrial version of the gene; Steele et al. 1996). It also requires that the gene which will be corrected by the presence of the transforming mitDNA carries a small deletion or a double mutation in order to avoid revertants. Linear DNA, in particular issued from PCR amplification product can also be used (Bonnefoy and Fox 2001).

10.3.1.2 Selection of Mitochondrial Transformants in a rho° Background

In most cases, the desired mitochondrial mutation is introduced in two steps (Fig. 10.3). First, a *rho*$^-$ synthetic strain carrying the mutation is constructed by biolistic transformation and in a

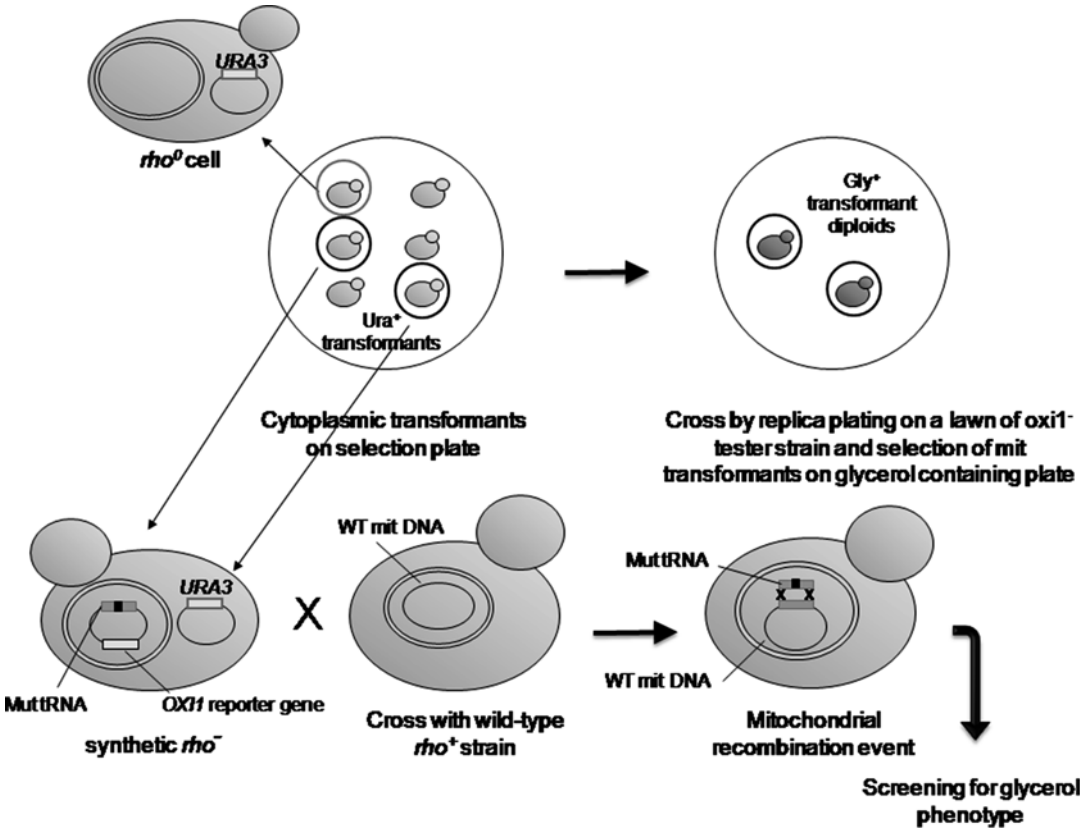

Fig 10.3 Schematic diagram for double selection of the transformants. In the first plate (ura$^-$ selective plate) are visualized colonies that have received the plasmid for nuclear selection. The transformants are crossed by replica plate with the tester oxi1$^-$ strain (TF145). Only the diploids that have received the "mitochondrial plasmid" bearing the reporter *OXI1* gene will grow on glycerol containing media. From the selective plate the haploids (called synthetic *rho*$^-$) are kept to perform the second cross with a wild-type *rho*$^+$ strain for mitochondrial recombination

second step the mutation is introduced by genetic cross into a wild-type mitochondrial genome by homologous recombination. This is the protocol of choice when no direct strong positive selection is available. In addition, it is sometimes worthy to keep the mutation as a synthetic *rho*$^{-}$ since it can then be transferred by crosses to various strains with different genotypes.

10.3.1.3 Selection of Survivors after Biolistic Transformation

As said previously, biolistic transformation is quite inefficient since more than 99 % of the cells are killed by the process. To circumvent this drawback, the first step done in any transformation is to select the surviving cells. This is performed by coating the particles with a normal replicative yeast plasmid bearing a selective gene marker such as URA3 for example, together with the "mitochondrial plasmid" (co-transformation). In a typical mitochondrial transformation experiment, a high number of *rho*° cells with a nuclear genetic marker (usually an auxotrophic phenotype such as [ura^{-}]) are bombarded by a large number of particles on a selective plate (minimal glucose supplemented for the other auxotrophies carried by the recipient strain). Some cells will survive to the hit and hence will grow on minimal medium (without uracile) and a small proportion of them will have the mitochondrial plasmid stably inserted into the mitochondria. With the optimal experimental conditions, it is quite easy to get 1,000–2,000 [ura+] colonies per plate. Figure 10.4 shows a schematic diagram of the plasmids used in a co-transformation experiment: the URA3 plasmid allows transformants to grow on selective medium (without uracile) whereas further genetic tests are necessary to identify which colonies, among the nuclear transformants, have also received the second plasmid into the mitochondria.

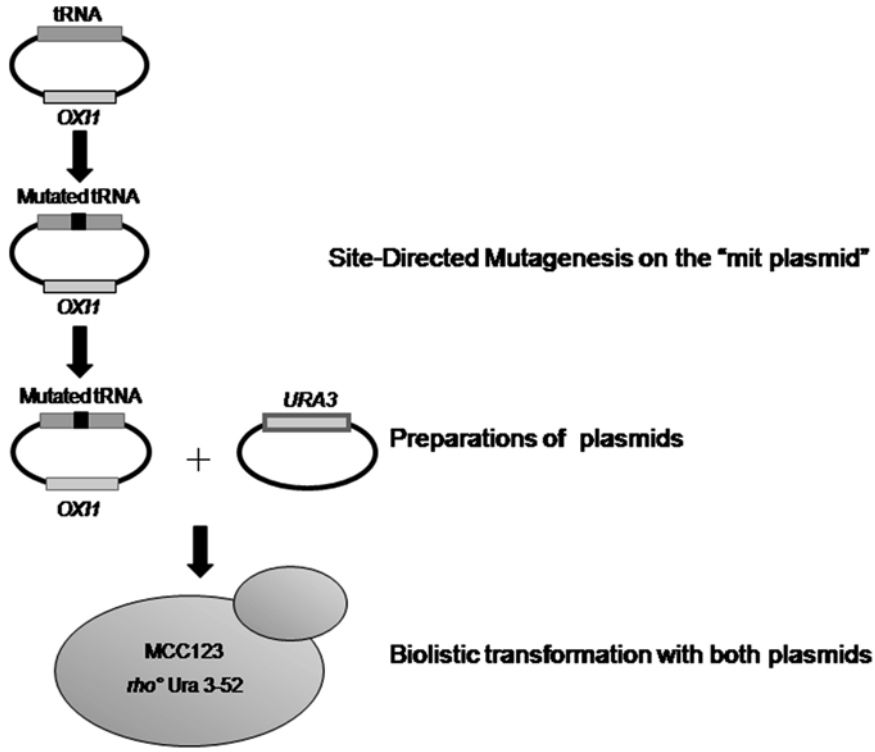

Fig 10.4 Scheme of the plasmids introduced via the biolistic co-transformation of a *rho*° strain

10.3.1.4 Selection of Mitochondrial Transformants

In a second step, the [ura+] transformants will then be individually checked for the presence of exogenous DNA into the mitochondria (mitochondrial transformants). Not all [ura+] cells contain the "mitochondrial plasmid" inserted within the mitochondria and correctly transmitted to the progeny. This "mitochondrial plasmid" presents several characteristics: (1) it does not carry any yeast replicative system so that, if not inserted into the mitochondria, it will be lost; (2) it contains a wild-type version of a mitochondrial-encoded reference gene (most often the OXI1 gene); (3) it contains an additional mitDNA fragment with the gene of interest carrying the desired mutation.

The mitochondrial transformants will then be selected among the nuclear transformants by a genetic test, crossing the putative synthetic *rho*$^-$ cells with a tester strain carrying a mutated form of the mitochondrially encoded control gene (usually oxi1) such as in the strain TF145 (MAT α, ade2-1, ura3-52, mit oxi1-17).

The presence of respiratory cells (gly+) among the diploids issued from the cross reveals that the wild-type allele of the OXI1 has recombined into the mutated oxi1 gene of the mitochondrial genome of the tester strain (Fig. 10.3). This indicates that the "mitochondrial plasmid" is localized within the mitochondrial compartment and that the original transformed colony is a synthetic *rho*$^-$. It is very important at this step to have carefully kept the corresponding haploid colony from the ura$^-$ selective plate since it is the synthetic *rho*$^-$ strain which will be used for further analysis. Frequency of positive clones will vary but usually 2–3 positive colonies are obtained per plate (remember that one plate equals one shoot and provides 1,000–2,000 [URA$^+$] colonies). Such haploid strains will have to be subcloned two or three times with the same method by crossing with the tester strain, in order to verify that they are stably transmitted to the progeny.

10.3.1.5 Integrating the Mutation into a *rho*$^+$ Genome

The synthetic *rho*$^-$ clone, once checked for its stability, can be stored indefinitely as any yeast strain and used for further genetic constructions. It can be crossed with a wild-type *rho*$^+$ genome or any appropriate genetic background *rho*$^+$ genome in order to express the mutation.

Since the homologous recombination process is frequent in yeast mitochondria, one should have a high percentage of recombinant strains which contain the new mitDNA (mutated) sequence integrated into the *rho*$^+$ genome. The frequency is described to be between 1 and 50 % (Bonnefoy and Fox 2007) but in our hands it has never been the case and the figure was closer to 0.1 %. It is known that crosses between *rho*$^-$ and *rho*$^+$ strains can yield much lower level of recombination than between *rho*$^+$ and *rho*$^+$ cross, depending upon the *rho*$^-$ structure and the position of the allele with respect to the mitDNA repetition (Bolotin-Fukuhara and Fukuhara 1976).

The experiment will therefore work all the better if a positive selection is available. The situation here is the same as with the direct transformation into a *rho*$^+$ strain (see above). One can select:(1) ability to grow on respiratory medium as described in Meunier (2001), for the COXI and COXIII subunits of cytochrome oxydase or in Wenz et al. 2007 for mutations in the Cytb gene; (2) inhibitor resistance; this phenotype has been used to identify biolistic transformants in yeast cytb (Fisher and Meunier 2005). However, it is important to note that drug resistance phenotypes, due to inhibitors to mitochondrial protein or ATP synthesis, are not ideal for use; in fact, resistant clones might arise spontaneously and can only be observed on fermentable medium in strains that respire; (3) [ARG$^+$] phenotype based on the Arg8^m synthetic gene. Arg8^m is an allotopically expressed nuclear gene, which codons have been modified to be expressed in the mitochondrial genome (Steele et al. 1996) and its expression is dependent on mitochondrial protein expression. Mitochondrial transformants are selected as arginine prototrophs which requires functional mitochondrial protein synthesis, absent in *rho*$^-$/*rho*$^\circ$ cells thus eliminating them from the screen. This marker has been used to allow the selection of any Cytb mutations independently of their functional or nonfunctional phenotype (Ding et al. 2008).

The situation is more complex if the desired mutation induces a very high proportion of secondary *rho*$^-$ clones such as ATP6 mutations, or if

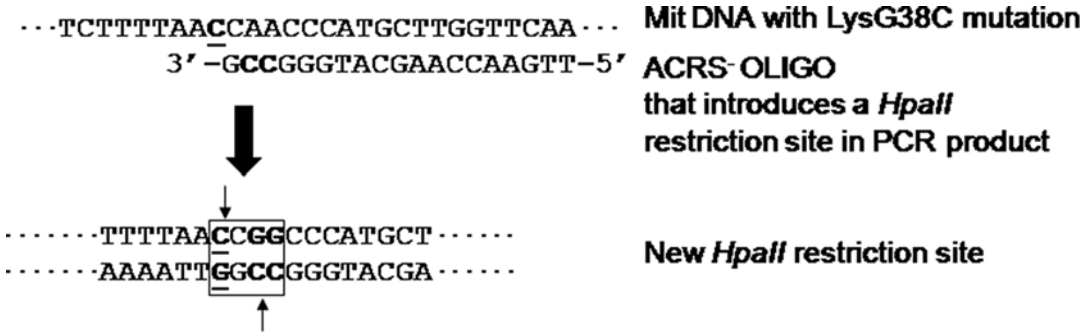

Fig 10.5 Example of ACRS strategy used for the selection of the tRNAlys mitochondrial transformants. The new HpaII site (*boxed*) is introduced by PCR primer with specific sequence (in *bold*) only in the presence of the mutated template (*underlined*)

one search for a negative phenotype such as tRNA mutations. The first case can be counter selected by using the Arg8^m gene which requires mitochondrial protein synthesis to be expressed eliminating all the *rho*$^-$ clones from the screening (Rak et al. 2007). In the case of tRNA mutations which by definition are defective for mitochondrial protein synthesis, this genetic trick cannot be used and we relied on molecular biology screens. The first mutants for which a strong defective respiratory phenotype was expected were screened by systematic sequencing of the tRNA gene of about 30 respiratory deficient colonies (Feuermann et al. 2003). Later on, in order to be independent of the mutant phenotype, an artificially created restriction site (ACRS).PCR technique was used on a large sample of random colonies (see Fig. 10.5). The ACRS.PCR technique involves a DNA polymerase chain reaction performed in transformant colonies in order to recognize those having acquired a single mitochondrial nucleotide substitution. The two oligonucleotide primers are chosen to amplify a region of about 100 bp containing the mutated DNA region. Only one oligonucleotide should be complementary to the single strand; the other one (ACRS) brought one or two base substitutions compared to the DNA sequence of the opposite strand so that a new restriction site will be generated into the amplification product. If and only if the mutation is present, the PCR fragment will carry this additional restriction site. Comparing the electrophoretic migration of the digested amplicon on 3 % agarose gel to an equal amount of undigested product allows to discriminate between wild-type (full length) and mutated DNA (two shorter fragments). PCR can be performed with pools of three–four colonies.

By chance, sometimes the desired mutation leads to a nucleotide change that introduces a new restriction site as compared to the wild type.

10.4 What Has Been Achieved with Yeast Biolistic Transformation?

The possibility to generate mutations in the mitochondrial genome has allowed progresses to be made on several aspects. For example, mutations in cyt b have been looked for with the objective of modelling the mammalian Qo site, or to further understand the differential efficacy of Qo site inhibitors on mammalian and pathogen bc1 complex (Fisher and Meunier 2005; 2008; Kessl et al. 2005; Ding et al. 2008; Vallières et al. 2013).

Reporter genes have been constructed and introduced into the mitDNA such as Arg8^m. This marker can be used for selection as pointed out previously and was the selective marker chosen for the first transformation made in another yeast than *S. cerevisiae*, the yeast *Candida glabrata* (Jingwen et al. 2010). It was also used as an expression marker to study diverse aspects of

mitochondrial processes (Steele et al. 1996; Sanchirico et al. 1998; He and Fox 1999; Bonnefoy and Fox 2000, 2001). A mitochondrially recoded version of GFP (GFP^m-3) also exists which can be used for similar types of studies (Cohen and Fox 2001).

Mutations having pathological equivalent in the corresponding human genes were also introduced in the COX1, COX3, or CYTB genes (Meunier 2001; Blakely et al. 2005; Fisher and Meunier 2001) and systematically looked for in the case of ATPase6. Five pathological mutations in ATPase6 have been modelled and studied in yeast (Rak et al. 2007; Kucharczyk et al. 2009a; 2009b; 2010; 2013) and their biochemical consequences deeply analyzed.

As for tRNA genes, from the first publication which showed that it was possible to recreate by biolistic transformation an already known random mutation (Rohou et al. 2001), to diverse mutations constructed later with diverse phenotypic effects and in various tRNA genes (Feuermann et al. 2003; De Luca et al. 2006; 2009; Montanari et al. 2014) about ten different situations have been analyzed. This set of mutations have been extremely useful to characterize the physiological and biochemical phenotypes of the mutants revealing the close parallel one can draw between human and yeast as far as mitochondrial genetics is concerned. They have also allowed to go a step forward toward therapy and search for suppressor effects either by correcting genes (Feuermann et al. 2003; De Luca et al. 2006; Montanari et al. 2010; 2013) or peptides (Francisci et al. 2011).

10.5 Conclusions and Perspectives

Biolistic transformation has proven particularly useful when no other transformation protocols worked efficiently, or at all. We can predict that no efforts will be made to develop this methodology for yeast nuclear transformation, even for yeasts for which no transformation has been done yet (electroporation is working efficiently in such situation) but it might be worth trying for some fungi, that are not easily manipulated. The transformation of fungi bearing biotechnological properties will allow a wide range of molecular and genetic experiments in order to study the physiological processes associated with their industrial interest.

The greatest interest of the biolistic transformation resides however in mitochondrial transformation. For many years, mitochondrial research practically only has been done with the yeast *S. cerevisiae* and biolistic transformation is nowadays a routine experimental technique, although still new screens can be developed and its efficiency slightly improved. However, other yeasts are now under scrutiny to exploit their very specific properties and in some case, mitochondrial functions are involved.

In addition, there is a new challenge: we now have at our disposal many yeast complete genome sequences with possibilities of innumerable comparative genomics studies. It will be very tempting, in addition to bioinformatic studies, to examine functional aspects of mitochondrial comparative genomics. The lack of suitable tools will be a serious handicap (as might be the lower homologous recombination efficiency). Mitochondrial transformation of *C. glabrata* shows nevertheless that some developments with new yeasts are possible and may open new research paths. Since no mitochondrial mutants are readily available in non *S. cerevisiae* yeasts, the authors (Jingwen et al. 2010) have used the $Arg8^m$ marker, which allows for a positive selection. The very interesting output of this experiment is that if mitochondria were indeed transformed the genetic information that was introduced did not integrate into the rho^+ genome and stayed within the mitochondria as heteroplasmic DNA. The degree of heteroplasmy, i.e. the fraction of mutant mitDNA coexisting with wild-type mitDNA in a cell is very important in the onset of mitochondrial pathologies (Chinnery et al. 2000); if the heteroplasmic state of the mitochondrial genome

proves to be a general phenomenon in *C. glabrata*, this will be a good tool to study heteroplasmy, as *S. cerevisiae* is not the most appropriate organism for such topic.

Of course *C. glabrata* is, like *S. cerevisiae*, a petite-positive (i.e. they can live without their mitochondrial genome) and fermentative yeast, while the challenge will certainly be greater with petite-negative respiratory yeasts. In such organisms, rather than destroying the mitochondrial function which is not possible, it might be of interest to add some genetic information. The non-availability of appropriate mitochondrial genetic markers may be overcome by a molecular screen; since the mitochondrial recombinants are about one per thousand nuclear transformants (in *S. cerevisiae*), it should be possible to detect the presence of specific mitochondrial sequences by colony hybridization if specific probes are available. In fact, such approach is being explored for *C. parapsilosis* (J. Nosek, personal communication).

We are convinced that in ten years mitochondrial reverse genetics will be available in other yeasts, our imagination being the limit.

10.6 Experimental Protocol

The protocol of Bonnefoy and Fox (2007) is the reference protocol and gives many important information. We have only introduced slight modifications to it.

10.6.1 Site-directed Mutagenesis

The site-directed mutagenesis is performed following the instruction manual of the QuickChange site-directed mutagenesis kit by Stratagene (Fig. 10.6). This kit was used to introduce point mutations in specific wild-type genes previously cloned in the pKS vector. Two mutagenic primers

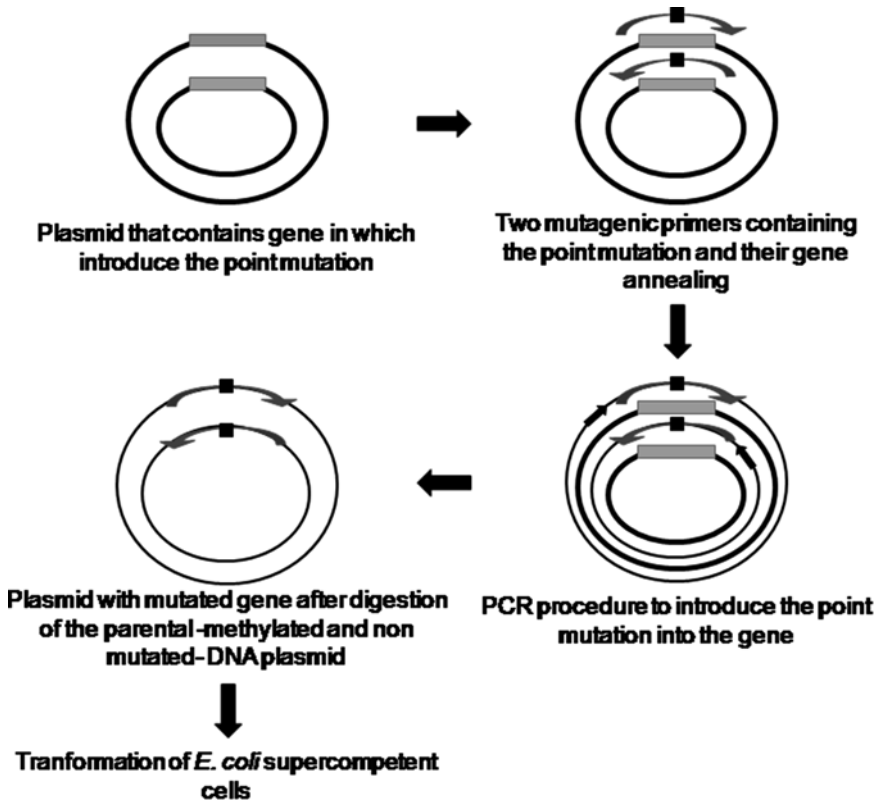

Fig 10.6 Site-directed mutagenesis steps; the gene interested by the procedure is indicated in *grey*. The mutation is indicated in *black square*

complementary to opposite strands of DNA, containing the desired mutation are used. The samples are prepared by adding to the template DNA, 125 ng of each oligonucleotide, 1 µl of dNTP mix, 5 µl of reaction buffer, 1 µl of Pfu Turbo polymerase (2.5 U/µl) and water to the final volume of 50 µl. The oligonucleotides are extended during PCR cycling by using Pfu Turbo DNA polymerase (5 min at 95 °C followed by 18 cycles: 1 min at 95 °C, 1 min and 30 s at 56 °C and 3 min at 68 °C, plus an extension of 9 min at 68 °C). The product is than treated by 1 µl DpnI. This enzyme digests the methylated and hemimethylated parental DNA. The resulted product has only the mutation-containing new synthesized DNA. This DNA is used to transform competent *E. coli* DH5α cells

10.6.1.1 Cell Preparation

The *rho°* strain to be bombarded is grown for 2–3 days (stationary phase) in 30–50 ml of YP (1 % yeast extract, 1 % peptone) containing 2 % galactose/0.1 % glucose from a fresh preculture. This amount is enough for about six shoots. The final yield is a little better when using raffinose which is quite expensive, but for efficient strains such as the MCC series, galactose is fine. One hour before the bombardment, cells are centrifuged and harvested to a concentration of $1–5 \times 10^9$ cells/ml and 100 µl of this suspension is plated on minimal glucose medium supplemented with 1 M sorbitol and amino acids to provide the appropriate prototrophic selection for the biolistic transformants.

10.6.1.2 Microprojectiles and Plasmid DNA Preparation

Tungsten or gold microparticles can be used but gold particles are rather expensive. If using tungsten, the Bonnefoy and Fox protocol calls for 0.7 µm particles (available from Bio-Rad) but we have noticed an improvement in efficacy by using a mixture (1/1) of 0.4 µm (Tungsten M-5) and 0.7 µm (Tungsten M-10) particles. In this case, two independent preparations of M-5 and M-10 particles in 1.5 ml 70 % ethanol are pre-sterilized in an Eppendorf tube by vigorous shaking (final concentration of 60 mg/ml), and incubated at room temperature for 10 min. The particles are washed with 1.5 ml of sterile water, resuspended in sterile frozen 50 % glycerol and kept on ice. This preparation can also be kept for months at −20 °C in 50 % glycerol and used directly after vigorous resuspension of the particles.

For six shots, mix in an Eppendorf tube: 5 µg of plasmid carrying the nuclear marker and a nuclear replication origin and 15–30 µg of plasmid carrying the mitochondrial DNA of interest (final volume of plasmid DNAs is 20 µl), then 50 µl of M-5 tungsten particles, 50 µl of M-10 particles, 4 µl of 1 M spermidine free base, and 100 µl of ice-cold 2.5 mM $CaCl_2$ and incubate the mixture for 15 min on ice with occasional vortexing. This preparation requires that everything be kept ice-cold (solutions are kept in the freezer till use), products are added in the order described and vortexed at each step. It is also important to note that the plasmid DNAs have to be highly concentrated and very pure. Preparations by ultracentrifugation or Quiagen columns work fine.

Spin the mixture briefly, 15 s at 13,000 rpm, and remove the supernatant. The particles are then resuspended in 200 µl of freezer-cold 100 % ethanol, first with pipette tip and then vortexing; the procedure was repeated several times until the particles were resuspended easily. At this stage, the particles are resuspended in 60 µl of cold 100 % ethanol and distributed by aliquots of 10 µl at the center of six macrocarriers placed in their holders, allowing the ethanol to evaporate.

10.6.1.3 Bombardement

The experiment is performed using a 1,100 psi rupture disk. The open petri dish carrying the lawn of cells was placed at 5 cm from the macrocarrier loaded in its holder into the assembly system. As Bonnefoy and Fox noticed, we also found that the yield was better when not assembling the stopping screens. The vacuum cham-

ber is evacuated until 27 mmHg is reached and the particles are shot. Any fragments of the macrocarrier disk were removed and plates were incubated at 28 °C for 4–5 days until colonies appeared.

10.6.1.4 Identification of the Mitochondrial Transformants

The plate containing the bombarded cells is replica plated on a lawn of the tester strain (TF145) containing a mit (oxi1-17) mutation on glucose-complete medium. It is incubated at 28 °C for 2 days and replica plated on 3 % glycerol containing medium. In glycerol containing media, only cells in which the mit mutation is complemented by the mitochondrial corresponding wild-type allele present on the "mitochondrial plasmid" are able to grow by respiration. Comparing the original bombarded plate and the plate after the cross, haploid colonies corresponding to the positions of respiring cells were picked (see Fig. 10.2). Quite often, it is difficult to identify an isolated colony at this stage but the test is repeated three times which allows subcloning and purification of a single cell colony. Store only the stable synthetic *rho*$^-$ clones, which transmit their allele to more than 80 % of the progeny.

Once the purified synthetic *rho*$^-$ is characterized, it can be stored and crossed with a *rho*$^+$ strain to allow recombination between the mutated gene and the wild-type gene present on the mitochondrial genome.

10.6.1.5 Interest of Cytoductants

The synthetic *rho*$^-$ strain has to be crossed to the appropriate *rho*$^+$ strain to construct the recombinant mit mutant. This can be done with a partner of the appropriate mating type and will yield diploids, some of which with the desired mutation. It is also sometimes interesting to screen directly into a haploid context, which can be done by cytoduction. If one of the strain (usually the synthetic *rho*$^-$) carries the *kar1-1* mutation (Conde and Fink 1976), the nuclear fusion is delayed while the mitochondrial fusion takes place as efficiently. It is therefore possible by selecting the genotype of the *rho*$^+$ partner to associate it to the synthetic *rho*$^-$ mit genome. Selection for the appropriate phenotype will be done as usual on the cytoductants population.

The *kar1-1* mutation is also useful to construct strains which are isomitochondrial with different defined nuclear genetic background. As an example we reintroduced the mutated mitochondrial genome obtained after sporulation of diploids (and therefore not isogenic) to the MCC123 nuclear background. Different biolistic mutants were crossed to MCC123 *(MATa, ade2-101, ura3-52, kar1-1*, and *rho°)* and four different cellular types could be distinguished: the original strains (MCC123 *rho°* and the biolistic mutants) the diploids and the new mutant with mutated mit DNA associated with MCC123 nuclear context (which we called M/mutant). The screening was facilitated by the presence of the ade2 mutation that enabled us to distinguish colonies with functional mitochondria, which accumulate a red pigment, from colonies with dysfunctional mitochondria, which appeared white (Kim et al. 2002). Figure 10.7 shows the schematic diagram of the colonies resulting from the cross between a Kar1-1, ade2, *rho°* and the ADE2 mit mutant strain.

When an ade2 strain is involved, the screening is very easy due to the fact that nonrespiring cells are not red as the wild type (as shown in the figure). However, it is possible to use other nuclear background. In this case the medium may contain adequate supplements as to select the haploid nuclear genome of interest. Since the red color cannot be used to screen for respiring/nonrespiring cells, one should use DAPI to confirm the distinction between *rho°* cells and defective respiring cells bearing the mutated mitDNA. De Luca et al. (2009) have shown that the severity of respiratory defects was highly variable depending on the different nuclear backgrounds.

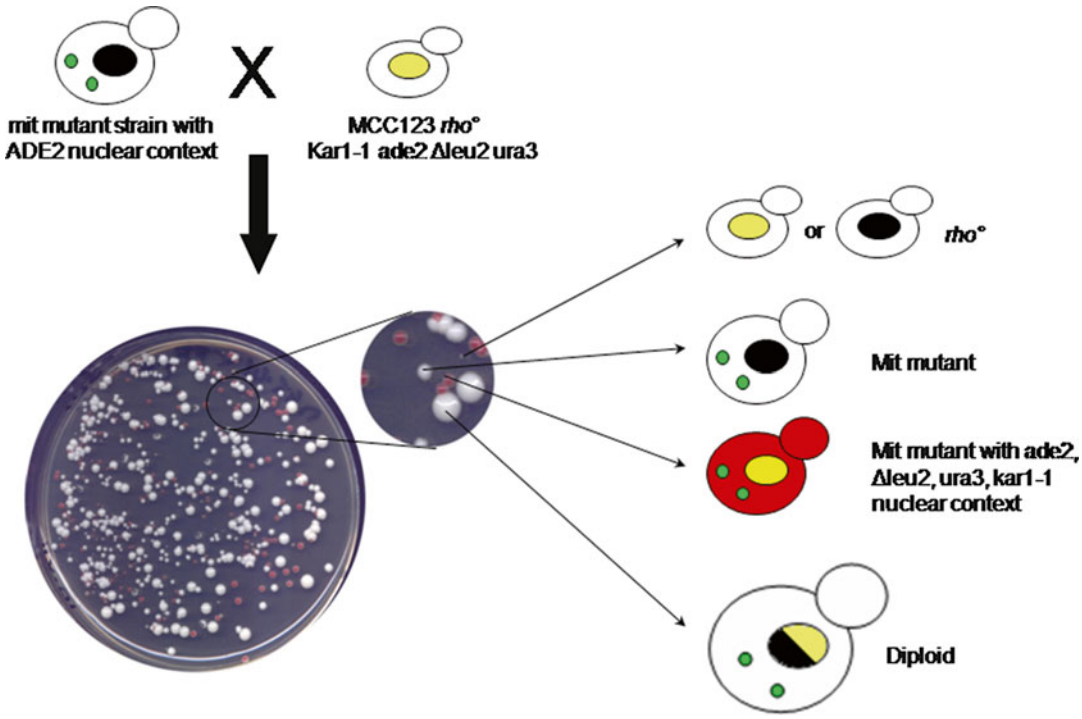

Fig 10.7 Schematic diagram of the colonies resulting from the cross between the Kar1-1, ade2, *rho*° and the ADE2 mit mutant strains

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11 Biolistic Transformation of *Candida glabrata* for Homoplasmic Mitochondrial Genome Transformants

Jingwen Zhou, Liming Liu, Guocheng Du, and Jian Chen

11.1 Introduction

Mitochondria are one of the most important organelles present in most of eukaryotic cells. A majority of key metabolic reactions, such as the citric cycle, ATP synthesis, amino acids synthesis, and fatty acids metabolism occur inside the mitochondrion (Foury et al. 1998; Talla et al. 2005; Koszul et al. 2003). Mitochondria can only reproduce themselves semiautonomously, during which many key reactions are dependent on gene products located on the nuclear genome (Ryan and Hoogenraad 2007; Falkenberg et al. 2007). Lack of mitochondria or mitochondrial genome, or deficiency in mitochondrial functions, could significantly change the metabolic phenotype of cells from yeast to human beings, such as slow growth or even cell death (Rak et al. 2007a; Clark-Walker 2007). Continuous investigations on mitochondrial functions revealed several correlations of the organelle with many important human diseases, making research on mitochondria a long-term hot spot in life science (Veatch et al. 2009; Harris et al. 2013).

Though there are many reports about those phenotypes or mechanisms related to mitochondria, only few of them could describe how to modify the genes on the mitochondrial genome (Cogliati et al. 2013; Avalos et al. 2013; Hughes and Gottschling 2012). According to our understanding of proteins encoded by genes located on the nuclear genome, manipulation of genes located on the mitochondrial genome should be the most direct route to fully understand their function and global impact (Rak et al. 2007b; Bonnefoy et al. 2007). Unfortunately, most of the previous studies on mitochondrial genomes are restricted to limited single cell system, such as *Saccharomyces cerevisiae*, *Candida glabrata*, and the green alga *Chlamydomonas reinhardtii* (Bonnefoy et al. 2007).

In most yeast species, mitochondrial genomes carry a series of key energy metabolism-related genes, such as *COX1*, *COX2*, *COX3*, *ATP6*, *ATP8*, and *ATP9*, and all mitochondrial tRNA genes (Foury et al. 1998; Talla et al. 2005; Koszul et al. 2003). Strains with mutated mitochondrial genome (ρ^-), or completely lacking mitochondrial genome (ρ^0) are respiration deficient and could form *petite* colonies (Strand et al. 2003). Cells that can grow without mitochondrial genome are referred to as "*petite*-positive", and those that are inviable without mitochondrial genome are termed "*petite*-negative". Most of the ρ^- and all of the ρ^0 cells are respiration deficient (Kominsky and Thorsness 2000).

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Mitochondria and mitochondrial genomes differ from the nucleus and the nuclear genome in several aspects, i.e., (1) the mitochondrion is generally smaller than the nucleus; (2) most eukaryotic cells have many copies of mitochondria and mitochondrial genome chromosomes (Falkenberg et al. 2007); and (3) codon usage of mitochondrial genes is different from nuclear genes. These differences make the genetic modification of mitochondrial genomes more difficult, hindering the usage of classical antibiotic resistance markers and biosynthetic markers (Adrio and Demain 2006; Zhou et al. 2009a). In 2009, Fox et al. developed a *ARG8*^{m} as the mitochondrial marker, which complements a nuclear Δ*arg8* deficiency in *S. cerevisiae* or *C. glabrata* when integrated into mitochondrial genome with a suitable mitochondrial promoter and terminator (McMullin and Fox 1993). *ARG8*^{m} contains two codons recognized as stop codons by the nuclear DNA (nDNA) system, therefore cannot complement when falsely integrated into the nucleic genome, or present in a yeast plasmid (Rak et al. 2007b; Bonnefoy et al. 2007).

A wide variety of defined alterations can now be generated in mitochondrial genomes, but this is limited to *S. cerevisiae* and *C. reinhardtii* (Bonnefoy et al. 2007). Few other successful systems for transformation of mitochondrial genome have been developed because of rigorous prerequisites. For example, to transform the mitochondrial genome of a *S. cerevisiae* strain, the strain should fulfill the following requirements, i.e., multiple auxotrophies (Δ*arg8*, Δ*ura3*/Δ*leu2*), and ρ^0 or ρ^+*mit*$^-$ respiratory phenotype. Among these prerequisites, *mit*$^-$ strains are highly difficult to obtain, while a ρ^0 strain can be easily obtained by culturing with ethidium bromide, the newly transformed ρ^0 cells must then be mated with ρ^+ cells after mitochondrial genome transformation (Bonnefoy et al. 2007). This is a nightmare for most of the yeast species or higher organisms.

Along with the development of biotechnology and medical science, more and more studies on non-conventional yeast species are reported. As one of the most important organelles, mitochondria and the mitochondrial genome are more and more studied (Rak et al. 2007a; Toogood 2008). The haploid facultative aerobe yeast *C. glabrata* (*Torulopsis glabrata*) is closely related to *S. cerevisiae* (Bialkova and Subik 2006) and has been studied for industrial biotechnological (Wang et al. 2005; Liu et al. 2004), clinical (Kaur et al. 2005; Polakova et al. 2009) and basic research applications (Schmidt et al. 2008; Muller et al. 2008). Contrary to other *Candida* species, *C. glabrata* is "*petite*-positive" (Chen and Clark-Walker 2000), i.e., it is viable in the absence of mitochondrial genome, making it an attractive model for genetic mitochondrial genome manipulation. The complete mitochondrial genome sequence of *C. glabrata* is 20 kb, which is the smallest among the sequenced hemiascomycetous yeast species (*S. cerevisiae*, 80 kb; *C. albicans*, 40 kb; *Yarrowia lipolytica*, 48 kb; *Pichia canadensis*, 27 kb) and similar in size to that of humans (Koszul et al. 2003).

Here, we use the *ATP6*, which is part of the mitochondrial genome of *C. glabrata*, and encodes subunit 6 (*a*) of the F_0 sector of mitochondrial F_0F_1-ATP synthase, as an example to show a different transformation and screening process of genetic operation on the mitochondrial genome of *C. glabrata* (Koszul et al. 2003). The *ATP6* gene was deleted from the mitochondrial genome of *C. glabrata* using DNA fragments containing a recoded *ARG8*^{m} mitochondrial genetic marker flanked by homologous regions to the target gene, delivered into mitochondria by biolistic transformation. Due to the multi-copy mitochondrial genome, the *ATP6* was partially deleted in *C. glabrata* mitochondrial genome heteroplasmic cells. With an extra anaerobic screening process, a homoplasmic Δ*atp6* strain was obtained from heteroplasmic transformants.

11.2 Materials

1. *C. glabrata* CCTCC M202019, a pyruvate overproducer (Liu et al. 2004). The *C. glabrata* Δ*arg8*Δ*ura3* double mutant was a derivative of the *C. glabrata* CCTCC M202019 (Zhou et al. 2009a). Other *C. glabrata* strains or similar haploid yeast strains without *ARG8* should also be suitable for the protocol.

2. Culture media: yeast peptone dextrose culture medium (YPD, 10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ peptone, 20 $g \cdot L^{-1}$ dextrose); yeast peptone glycerol culture medium (YPG): 10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ peptone, 20 $g \cdot L^{-1}$ glycerol; minimal medium (MM): 20 $g \cdot L^{-1}$ glucose, 1.0 $g \cdot L^{-1}$ KH_2PO_4, 0.5 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 10 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, 100 $mg \cdot L^{-1}$ uracil; supplement medium with arginine (SM): MM with 100 $mg \cdot L^{-1}$ arginine. MM-S and SM-S were MM and SM with 1 $mol \cdot L^{-1}$ sorbitol. The initial pH of all medium was adjusted to 5.5. All media included 10 mL of vitamin solution (1.0 $g \cdot L^{-1}$ niconacid, 5.0 $mg \cdot L^{-1}$ biotin, 5.0 $mg \cdot L^{-1}$ vitamin B_1, 50 $mg \cdot L^{-1}$ vitamin B_6, filter sterilized). All plates were the corresponding liquid medium with 15 $g \cdot L^{-1}$ of agar.
3. Sterile distilled water.
4. Vortexer.
5. Pipettes and tips.
6. Ethanol (70 and 100 %).
7. Microcentrifuge with temperature control, e.g., Eppendorf 5410R.
8. Disposable polypropylene microcentrifuge tubes: 1.5 mL conical; 2 mL screw-capped.
9. PCR tubes.
10. TE: 10 mM Tris–HCl pH 7.5 (25 °C), 0.1 mM EDTA.
11. Thermostable DNA polymerase (e.g., Takara ExTaq (Code No. RR001A) with dNTP and Mg^{2+} solution). Any DNA polymerase is OK.
12. Oligonucleotide primers.
13. Thermal cycler, e.g., C1000 Touch (Bio-Rad).
14. Real-time thermal cycler, e.g., LightCycler 480 II (Roche).
15. Agarose.
16. TBE buffer: 50 mM Tris, 50 mM boric acid, 1 mM EDTA. Dilute when needed from a 10× stock.
17. Ethidium bromide, golden view, or any other dyes for DNA electrophoresis.
18. Gel loading mixture: 40 % (w/v) sucrose, 0.1 M EDTA, 0.15 mg/mL bromophenol blue.
19. Horizontal electrophoresis equipment (e.g., Bio-Rad Wide Mini Sub Cell).
20. U.V. transilluminator and camera suitable for photographing agarose gels, e.g., Gel Doc XR system (Bio-Rad).
21. Biolistic PDS-1000/He Particle Delivery System (Bio-Rad).
22. 0.4 μM tungsten particles (available from Bio-Rad, Cat. Nos. 165-2265).
23. CO_2 Cell Culture Incubator with controllable nitrogen gas or CO_2 supply, e.g., MCO-18AIC (Sanyo. Optional.). This can also be replaced by a simple sealed container that could be filled with nitrogen gas.
24. Sterile console.
25. Anaerobic sterile console (optional).
26. Real-time PCR kits with SYBR green [e.g., SYBR Premix Ex Taq™ II (Takara, Dalian, China)].

11.3 Methods

11.3.1 Preparation of Competent Cells for Biolistic Transformation

This method has been used to prepare competent *C. glabrata* cells for biolistic transformation.

1. *C. glabrata* cells were streaked on YPD plates and incubated for 48 h to form single colonies.
2. *C. glabrata* cells were cultured (200 rpm, 30 °C) in YPD culture medium from single colonies obtained above to an OD_{600} of 1.0–1.2. This represents a cell density of approximately $1.0–1.2 \times 10^7$ cells/mL. (One needs 100 mL per transformation in a 500 mL shake flask, so prepare 3–5 shake flasks each time).
3. Harvest cells by centrifugation at $4{,}000 \times g$ for 5 min at 4 °C.
4. Cool down the cells on ice for 5 min.
5. Wash cells with 100 mL of cold (4 °C) MM-S by centrifugation for three times.
6. Concentrate 100–200 times in MM-S to reach a cell density of $1–5 \times 10^9$ cells/mL and split the solution into 0.5 mL aliquots.
7. One 0.5 mL cell aliquot cell was spread onto MM-S plates for pre-cooling at 4 °C before biolistic transformation (Bonnefoy et al. 2007).

11.3.2 Construction of Vectors for Mitochondrial Genome Transformation of *C. glabrata*

This method has been used to construct a vector for the mitochondrial genome transformation of *C. glabrata*. To ensure the identification of transformants, the vector should contain a mitochondrial marker with a promoter that can work inside mitochondria. Here, we used an *ARG8* ^{m} gene, which is an essential gene for arginine biosynthesis with a mitochondrial codon, to replace a mitochondrial gene, *ATP6*. The correct replacement of *ATP6* with *ARG8*^{m} will result in *ARG8*^{m} under control of the *ATP6* promoter.

1. The plasmid pDS24 containing an *ARG8*^{m} gene (Steele et al. 1996) was used as template for PCR amplification of Δ*atp6*::*ARG8*^{m} cassettes with primers Con-ATP6-F (GCggatccAATATTATTTATTATATAATAA TATTAATTTTAATAAGTTATAATATA TATTTATAAAGT**ATGACACA TTTAGAAAGAAG**) and Con-ATP6-R (GCG ggatccTATTAATAATAATTAATTAAA GAATATTATAATATAATTAATTTAT TTGTATTATATAAA**TTAAGCATAT ACAGCTTCG**).
2. The primers contained a *Bam*HI site at their 5′-end and regions of homology to *ATP6* and *ARG8*^{m}. The regions of homology (bold and underlined) to *ATP6* ORF comprised 60 bp upstream of the *ATP6* initiation codon and 60 bp downstream of the *ATP6* stop codon, respectively (Rak et al. 2007b).
3. PCR products were digested with *Bam*HI and inserted into pUC19. The resulting plasmid was named pUC-atp6::ARG8m.

11.3.3 Preparation and Coating of Tungsten Particles

This method has been used to prepare and coat tungsten particles with DNA samples for the biolistic transformation.

1. Plasmid or DNA fragments were extracted, purified with an EZ Spin Column Plasmid Medi-Preps Kit (Bio Basic Inc., Markham, Canada), and concentrated to 2 μg μL^{-1} with DNAMate (Takara, Dalian, China).
2. The DNA concentration and purity of plasmids for transformation was determined with a NanoDrop 2000c (Thermo Fisher, Wilmington, DE).
3. Sterilize 50 mg of 0.4 μm tungsten particles by suspension in 1.2 mL of 70 % ethanol in a microfuge tube and incubation at room temperature for 10 min. Repeat for two times.
4. Wash the particles with 1.5 mL of sterile water and resuspend at 60 mg/mL in sterile 50 % glycerol. We recommend to use fresh prepared particles to prevent potential caking.
5. Take 20 μg of plasmid or DNA fragment (here we use pUC-atp6::ARG8m) carrying the mitochondrial DNA of interest with suitable markers (here we use *ARG8*^{m}), in a total volume of 15–20 μL.
6. Add and mix 100 μL of tungsten particles, 4 μL of 1 M spermidine-freebase, and 100 μL of ice-cold 2.5 M $CaC1_2$ (Bonnefoy et al. 2007).
7. Incubate for 10 min on ice with occasional vortexing.
8. Centrifuge shortly and remove the supernatant.
9. Resuspend the particles thoroughly in 200 μL of 100 % ethanol with vigorous shaking or pipette to prevent aggregation of particles.
10. Centrifuge slightly to remove the supernatant.
11. Add 50 μL of 100 % ethanol.
12. Distribute the particles in 100 % ethanol evenly at the center of five macrocarriers (flying disks) placed in their holders, allowing the ethanol to evaporate.

11.3.4 Biolistic Transformation Process

This method has been used to transform the *C. glabrata* mitochondrial genome by biolistic gun.

1. Sterilize the whole biolistic transformation system and essential tools, in advance, by UV for 30 min.

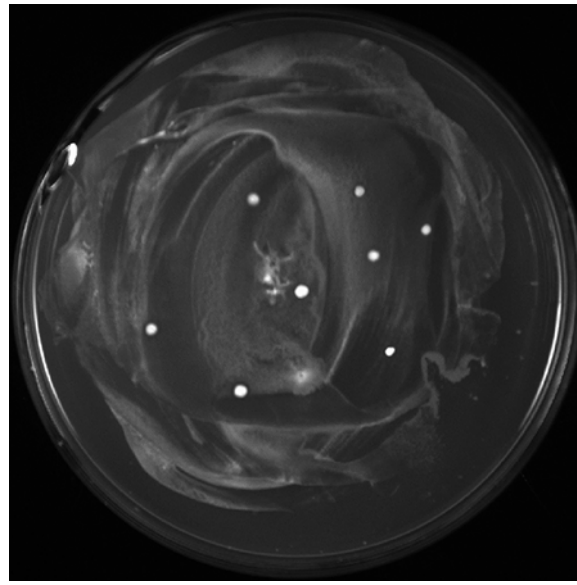

Fig. 11.1 A typical plate by biolistic transformation. *C. glabrata* Δ*arg8*Δ*ura3* double mutant was transformed with pUC-atp6::ARG8m bybiolistic gun using a PDS-1000/He System (Bio-Rad, Hercules, CA) on MM plate. The transformed plate was cultured under anaerobic condition at 30 °C for 72 h. The crater is at the center of the plate. The pattern of the transformants did not follow the Poisson's distribution. (From Zhou, J. W., Liu, L. M., Chen, J. (2010) Mitochondrial DNA heteroplasmy in *Candida glabrata* after mitochondrial transformation. © American Society for Microbiology, Eukaryot. Cell, Vol. 9, No. 5, 2010; p. 806–814, doi:10.1128/EC.00349-09 with permission)

2. Rupture disks of 900, 1,100, and 1,350 psi were used for yeast mitochondrial genome transformation and a stopping screen was not assembled (Butow et al. 1996).
3. Distances between the disk with the lawn of cells and the macro carrier assembly were 6, 9, and 15 cm.
4. Biolistic transformation was performed using a PDS-1000/He System (Bio-Rad, Hercules, CA) (Bonnefoy et al. 2007) and bombarded plates were incubated at 30 °C for 3–4 days until colonies appeared.
5. Transformants were identified as Arg^+ prototrophic colonies and sub-cloned on MM-S for at least three generations (Fig. 11.1).

11.3.5 Identification of Heteroplasmic Phenotype of Mitochondrial DNA Transformants

Since there are multiple copies of the mitochondrial genome in one cell under most of conditions, it is possible that two or even more types of different mitochondrial genomes could exist in one single cell (Druzhyna et al. 2008; Berger and Yaffe 2000; Kang and Hamasaki 2002). This phenomenon is termed as the mitochondrial genome heteroplasmy and can occur in yeast and higher organisms (Shitara et al. 1998; Burgstaller et al. 2007; Sachadyn et al. 2008). Often heteroplasmy is not a stable state. However, it was found that

some plant and animal cells could maintain a heteroplasmic state for extended periods (Hanson and Folkerts 1992; Wallace 1992).

Biolistic transformation of *C. glabrata* could not avoid the mitochondrial genome heteroplasmy since one cannot transform each copy of the mitochondrial genome in one cell. In mice, single cells with multiple different mitochondrial genomes tend to eliminate specific mitochondrial genomes (Shitara et al. 1998; Gyllensten et al. 1991). Heteroplasmic *S. cerevisiae* cells are not stable under most conditions (Lewin et al. 1979) because they rapidly become homoplasmic through mitotic segregation (Bonnefoy et al. 2007), during which new buds receive relatively few mitochondrial genome copies from mother cells due to highly asymmetric *S. cerevisiae* budding (Zinn et al. 1987). Since *C. glabrata* typically divides symmetrically, which is different from *S. cerevisiae*, the maintenance and elimination of heteroplasmy in *C. glabrata* is different. Besides, no mating process was observed in *C. glabrata* (Muller et al. 2008), further hindering the elimination of heteroplasmy by mating.

1. Half of a single colony, or 20 μL of cultured cells was transferred to a 1.5 mL-Eppendorf tube and heated in a microwave oven for 1 min (600 W) before adding 25 μL of premixed PCR-reaction mixture. PCR was carried out as 94 °C for 4 min for one cycle; then 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min per kb, for 30 cycles, followed by 10 min at 72 °C (Duenas et al. 1999). The replacement of *ATP6* and existence of *ARG8*^{m} were validated by primer pairs ATP869-F (CACTATTGGT GGTATGACAG)/ATP869-R (GTGTTGGC ACATCATTACTA) and ARG8m-F (ATGAC ACATTTAGAAAGAAG)/ARG8m-R (TTAAGCATATACAGCTTCG), using exTaq (Takara, Dalian, China).
2. Correct transformants were picked, grown in 20 mL of MM (250 mL flask) for 24 h (200 r·min^{-1}, 30 °C), washed (4,000×*g* for 1 min) with MM, and grown under specific conditions.
3. Anaerobic growth conditions were achieved by perfusion with purified nitrogen gas. Cells were washed with sterilized MM, diluted (10^{-5}, 10^{-6} and 10^{-7}), and spread on MM.
4. The colonies that appeared on MM after 72 h were transferred to corresponding YPD and YPG plates.
5. The percentage of heteroplasmic cells was calculated by the ratio of colonies on YPG to colonies on YPD.
6. To avoid the interference of nDNA, the mitochondrial genome was purified as described (Defontaine et al. 1991). Potential residual linear nDNA was eliminated by digestion with λ-exonuclease and RecJ$_f$ (New England Biolabs, Ipswich, MA) at 37 °C for 16 h, and inactivated at 65 °C for 10 min (Balagurumoorthy et al. 2008). The λ exonuclease is an exodeoxyribonuclease that digests double-stranded DNA from the 5′ end and forms single-stranded DNA (Subramanian et al. 2003), while RecJ$_f$ is a single-strand-specific exonuclease that digests the remaining complementary single strand to mononucleotides (Lovett and Kolodner 1989). Combining the two removes linear DNA from a mixture of linear and supercoiled DNA, leaving the supercoiled mitochondrial genome intact (Balagurumoorthy et al. 2008).
7. Cultures were further incubated and diluted with fresh medium to prevent cell densities in excess of 5×10^{7} cells mL^{-1}, and to maintain exponential growth.
8. Numbers are the percentage of Arg^{-} clones that could grow on YPD or SM, but not MM, to Arg^{+} clones. At least 500 colonies were counted for each condition.
9. The relative mitochondrial genome copy number (RCN) was determined by quantitative PCR (qPCR) with SYBR Green (Taylor et al. 2005). Nucleic (qPCR-ACT1-F (5′-AGTTGC TGCTTTAGTTATTG-3′)/qPCR-ACT1-R (5′-CTTGGTGTCTTGGTCTAC-3′ (Muller et al. 2008) and mitochondrial genome primers (qPCR-COX1-F (5′-TGA GAACTAATGGTATGACAATGC-3′)/qPCR-COX1-R (5′-GTAACACCTGCTGATAATAC TGG-3′)) were used to amplify *ACT1* and *COX1* with SYBR Premix Ex Taq™ II (Takara, Dalian, China). PCR reactions were performed on a Bio-Rad iCycler and analyzed with iCycler IQ software Version 3.0a (Bio-Rad, Hercules, CA). At least three experi-

ments were run for each condition analyzed. The relative amounts of *ATP6* and *ARG8*^{m} were determined with a qPCR assay similar to that used for RCN. *ATP6* and *ARG8*^{m} were amplified with primers qPCR–ATP6-F (5′-CTTATGTTGCTAGAGCTTTCT-3′)/ qPCR-ATP6-R (5′-AATACCAAATTCTAAG CACAT-3′) and qPCR-ARG8m-F (5′-CAC CAGTTGTACTACGAAGTTCTC-3′)/ qPCR-ARG8m-R (5′-TGATAAAGCACCCA TTGTTCTACC-3′).

11.3.6 Screening of Homoplasmic Mitochondrial DNA Transformants

Host *C. glabrata* cells used here were Δ*arg8* and could not synthesize arginine, making the arginine-synthesizing ability of mitochondrial genome without *ATP6* (mtDNA(Δ*atp6*::*ARG8*^{m})) essential, and forcing the maintenance of mtDNA(Δ*atp6*::*ARG8*^{m}). Therefore, although a large number of cells selectively lost mtDNA (Δ*atp6*::*ARG8*^{m}) under aerobic conditions, a group maintained mtDNA(Δ*atp6*::*ARG8*^{m}). This could be caused by that both mitochondria with wild-type mitochondrial genome (mtDNA (*ATP6*)) and mtDNA(Δ*atp6*::*ARG8*^{m}) fail to generate ATP from the oxidative phosphorylation during anaerobic growth (Zhou et al. 2009b). Thus, the selective advantage by efficient ATP synthesis was eliminated. This increases the viability of cells with mtDNA(Δ*atp6*::*ARG8*^{m}) during anaerobic growth.

In previous studies, it was found that the mitochondrial genome copy number in *C. glabrata* cells can decrease to a very low level during anaerobic growth (Zhou et al. 2010). This repression of mitochondrial biogenesis influences the ratio of the two different mitochondrial genomes. The decreased mitochondrial number could facilitate the occurrence of homoplasmic cells. Anaerobic cultivation could affect the mitochondrial genome maintenance on two aspects: (1) Under anaerobic conditions, F_0F_1-ATPase cannot produce ATP; (2) Anaerobic growth could inhibit mitochondrial biogenesis, thus decreasing the mitochondrial genome copy number. Repression of mitochondrial biogenesis under anaerobic conditions further decreased the copy number of mtDNA (*ATP6*). This led to an increased loss of mtDNA (*ATP6*) in some heteroplasmic cells (Berger and Yaffe 2000).

Based on the two aspects, the following method facilitates screening homoplasmic mitochondrial genome transformants.

1. Verified transformants on sub-cloned plates were picked, grown in 20 mL of MM (250 mL flask) for 24 h (200 r·min^{-1}, 30 °C), washed (4,000×*g* for 1 min) with MM and grown under anaerobic growth conditions. Anaerobic growth conditions were achieved by perfusion with sterile nitrogen gas.
2. Cells were washed with sterilized MM, diluted (10^{-5}, 10^{-6} and 10^{-7}), and spread on MM.
3. The colonies that appeared on MM after 72 h were transferred to corresponding YPD and YPG plates.
4. The replacement of *ATP6* and existence of *ARG8*^{m} were validated by primer pairs ATP869-F (CACTATTGGTGGTATGACAG)/ ATP869-R (GTGTTGGCzACATCATTACTA) and ARG8m-F (ATGACACATTTAGAAAG AAG)/ARG8m-R (TTAAGCATATACAGCT TCG), using exTaq (Takara, Dalian, China). Those with only *ARG8m* bands were the potential homoplasmic mitochondrial genome transformants. Homoplasmy could be further confirmed by Southern blot or other experiments.

11.4 Notes

1. According to our experience with both *C. glabrata* and *S. cerevisiae*, biolistic transformation efficiency of the mitochondrial genome is very low. Therefore, during preparation of competent cells, make sure that there are relatively thick layers on the plates (Zhou et al. 2010). To prevent the movement of the wet competent cells, the plates could be dried a little in a sterile console by wind. Do not worry about too many transformants, since most times you can just get very few transformants.

A very thin layer of competent cells similar to the amount for electroporation or chemical transformation process does not work. We think that the thicker layer could facilitate the absorbance of more particles.

2. Heteroplasmic mitochondrial genome transformants with mitochondrial genome that are deficient in energy metabolism genes (actually, a majority of genes located on mitochondrial genome are related to this) are highly sensitive to oxygen. Mitochondrial genome with deficiencies could be lost at a very high frequency even with the screening pressure of arginine. Addition of oxidative phosphorylation inhibitors, such as oligomycin, dicyclohexylcarbodiimide (DCC), or dinitrophenol (DNP) could release the process. We recommend that all of the experiments with heteroplasmic transformants should be performed under anaerobic conditions.
3. Even as a homoplasmic transformants, loss of mitochondrial genomes that are deficient in energy metabolism genes could also occur as a very high frequency even with the screening pressure of arginine. Restreaking the transformants on plates without arginine from time to time is essential. Our suggestion is that one could finish the related experiments on it in a shorter time.

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Use of the Biolistic Particle Delivery System to Transform Fungal Genomes

12

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12.1 Introduction

Genetic transformation systems have been developed for both ascomycetous and basidiomycetous fungi including gilled basidiomycetes, thus making possible the genetic modification of industrial protein producers as well as fungi targeted for food and biological control applications. A "universal" transformation method successfully applied for a large number of filamentous fungi is polyethylene glycol-mediated DNA uptake by protoplasts. Other previously used methods include electroporation of protoplasts (Goldman et al. 1990) and incubation of germinating conidia in a lithium salt (Dhawale et al. 1984). In addition, a relatively high frequency transformation of both fungal conidia and protoplasts has been reported using *Agrobacterium* T-DNA (de Groot et al. 1998). The biolistic delivery system (gene gun) was initially applied to introduce genetic material into plant cells (Klein et al. 1987, 1988; Sanford 1988, 1990), but the use of this technology has spread widely into other cell types, including neuronal tissue (O'Brien et al. 2001), stem cells (Uchida et al. 2009), and filamentous fungi. Examples of fungal species of which the genome has been transformed by biolistic bombardment include "academic" fungi such as *Aspergillus nidulans* (Herzog et al. 1996) and *Neurospora crassa* (Armaleo et al. 1990) and the high protein secreting industrial "workhorse" *Trichoderma reesei* (Hazell et al. 2000; Te'o et al. 2002), amongst others.

Nucleic acid material to be transformed and integrated into the targeted host genomic DNA is coated onto inert heavy metals as tungsten or gold, using calcium chloride and spermidine, facilitated by incubating the mixture on ice. The choice of using either gold or tungsten is usually made by looking into the cost involved and the material to be bombarded; gold particles are more expensive but smoother than tungsten may cause less damage to the target cells. The microparticles coated with DNA are accelerated at high velocity under vacuum onto the target cells (e.g., fungal conidia) plated at the center of an agar plate placed facing up at a designated "shooting" target distance of 3, 6, or 9 cm, preset in the bombardment chamber. The further away the target distance is, the wider the spread of microparticles and the optimal acceleration velocity for the microparticles becomes important (Te'o et al. 2002). The basic PDS-1000/He system sold by the company Bio-Rad (Bio-Rad Laboratories, Inc, http://www.bio-rad.com/) contains a single barrel for particle delivery. Also handheld devices are available albeit not generally used to bombard fungi.

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Using the filamentous fungus *T. reesei* as an example, the plate containing fungal conidia bombarded with microparticles coated with DNA is removed from the chamber and incubated at 28 °C for a minimum of 2 h to allow for cell recovery, before the addition of either water or saline solution to help spread the conidia to cover the entire agar plate before returning the plate back at 28 °C for at least 4 h. An overlay agar containing the selection antibiotic (e.g., hygromycin B at a suitable final concentration) is prepared and added to cover the fungal conidia; the plate will be incubated further at 28 °C until transformant colonies start appearing. Typically after 3 days, small colonies start emerging and after 5 days the larger colonies can be picked and patched onto new agar plates containing the antibiotic. After incubation at 28 °C for about 5 days, only "true" transformants will survive.

In contrast to high numbers of transformants produced using fungal protoplasts (Penttilä et al. 1987), much lower numbers have been reported using the "single-barrel" gene gun method (Hazell et al. 2000; Miyauchi et al. 2013). Higher numbers-up to 50 transformants per plate-can be generated using the Hepta Adaptor particle delivery system carrying seven barrels-an option provided by Bio-Rad (Bio-Rad Laboratories, Inc, http://www.bio-rad.com/). In this case seven lots of conidia are plated evenly on the agar plate to be aligned with the Hepta Adaptor device before transformation. In general, the transformation frequencies with filamentous fungi, notwithstanding the method used, are typically in the range of 10–100 transformants per microgram of DNA. It is worth noting that biolistic bombardment also allows co-transformation, where microparticles coated with different DNAs are mixed (e.g., 2–5 μg of each DNA) and the cells bombarded with this mixture. Hazell et al. reported a co-transformation efficiency of 92 % using hygromycin B selection (Hazell et al. 2000).

Newly created recombinant fungal strains with their genomes transformed using the gene gun approach have been reported to contain at least four copies of foreign DNA stably integrated in their genomes (Te'o et al. 2000) or 10–30 "functional plasmids" present per transformant (Armaleo et al. 1990). Both linear and circular DNA have been used for the transformation by particle bombardment. For targeted integration, the transforming DNA contains flanking homologous DNA fragments of about 1 kb in size, to aid in homologous integration at the locus of interest in the host genome (Miyauchi et al. 2013; Te'o et al. 2000). Transformant stability can be maintained by propagating the recombinant fungal strain on an agar medium containing the appropriate antibiotic.

12.2 Materials

1. Sterile distilled water
2. Potato dextrose agar (PDA)
3. Petri dishes (e.g., 9 cm in diameter, sterile)
4. Fungal conidia (prepared as instructed, see Sect.12.3.1)
5. Antibiotic for transformant selection (e.g., hygromycin B)
6. Ethanol (100 %)
7. Ethanol wash bottle (70 % v/v)
8. Glass spreader
9. Hemocytometer (e.g., Neubauer Chamber, 30×70 mm thickness)
10. Glass funnel with cotton plug (use wettable cotton)
11. M Spermidine
12. M $CaCl_2$
13. Double-stranded DNA (circular or linearized)
14. 0.9 % w/v NaCl, 0.01 % v/v Tween 80 for resuspension of conidia
15. Gold (0.6 μ) microcarrier particles (e.g., Bio-Rad #165-2262), or
16. Tungsten (0.7 μ) microcarrier particles (e.g., Bio-Rad #165-2266) [1]
17. Macrocarriers (e.g., Bio-Rad #165-2335)
18. Macrocarrier holders (e.g., Bio-Rad #165-2322)
19. Rupture disks, 650 psi (e.g., Bio-Rad #165-2326) [2]
20. Stopping screens (e.g., Bio-Rad #165-2336)
21. The PDS-1000/He System (Bio-Rad #165-2257) [3]

22. Vacuum pump (e.g., JAVAC DD300)
23. Helium tank and regulator (High purity)
24. Microcentrifuge

12.3 Methods

The following protocols are especially developed for *T. reesei* but can be adapted to other filamentous fungi as well, in consultation with the footnotes provided.

12.3.1 Preparation of Conidial Solution for the Bombardment

The age of fungal conidia is an important consideration for their transformation. With *T. reesei*, up to two weeks old conidia are suitable for the bombardment. A dark green color of conidia indicates that they are ready for harvesting.

1. Prepare plates containing 20 mL PDA. When PDA is set, dry plates (e.g., for 6–7 min in a 70 °C oven).
2. Spread fungal conidia onto PDA plates and incubate at 28 °C for growth and production of fresh conidia (e.g., 1–2 weeks).
3. When ready, add 5 mL of 0.9 % w/v NaCl, 0.01 % v/v Tween 80 onto the PDA plates, gently remove conidia by scraping with a sterile glass spreader.
4. If necessary, filter the conidial solution into a clean sterile test tube through glass funnel containing cotton plug to remove any hyphae.
5. Prepare a conidial solution by diluting in water (e.g., 1:50). Use hemocytometer to count conidial concentration.
6. Spot 1×10^7 conidia from step 4 in the center of PDA plate(s).[4] Leave plates to dry at room temperature (RT).

12.3.2 Preparation of Microparticles and Precipitation

The type and size of microparticles depend on the target cells (*11*, *13*). The starting amount of gold or tungsten particles given in the protocol is sufficient to transform conidia plated on five PDA plates.

1. Weigh 50 mg of either gold or tungsten microparticles into a 1.5 mL Eppendorf tube.
2. Wash microparticles with 1 mL absolute Ethanol. Vortex, collect (e.g., 10,000 rpm, 5 s), remove Ethanol. Repeat two times. After the final wash in Ethanol, add 1 mL distilled sterile water, vortex, collect briefly (e.g., 10,000 rpm, 5 s), and remove water. Resuspend particles in 1 mL of fresh water; the particles are now ready for use.[5]
3. Vortex the tube containing microparticles, and transfer a 50 μL sample to a new Eppendorf tube.
4. Add dsDNA (>2.5 μg). Vortex to mix (>30 s).
5. Add 50 μL of 2 M $CaCl_2$. Vortex to mix (>30 s).
6. Add 20 μL of 0.1 M Spermidine. Vortex to mix.[6]
7. Incubate the tube that contains microparticles coated with dsDNA on ice for at least 30 min.
8. After incubation, spin the tube for 10 s (e.g., 6,000 rpm) and discard supernatant.
9. Wash DNA-coated microparticles with 500 μL absolute Ethanol. Flick the tube with finger to resuspend particles and DNA. Spin down (e.g., 6,000 rpm, 5 s) and discard supernatant.
10. Add 60 μL Ethanol (absolute), mix by flicking the tube with finger.
11. Pipette 10 μL onto the center of each macrocarrier prepared as described below. Leave to dry.
12. As a control, process microparticles as described above but without DNA.

12.3.3 Preparation of the Gene Gun and Biolistic Transformation

1. Clean the PDS-1000/He System by spraying the chamber with 70 % Ethanol and wipe to dry. Place five macrocarriers in Ethanol and place each separately in the macrocarrier holders to dry. [3, 7]

2. Turn on vacuum pump to warm up.
3. Assemble the PDS-1000/He System as follows: (a) dip a 650 psi rupture disk in 100 % Ethanol to sterilize and place it at the bottom of the Helium chamber to temporarily stop Helium gas buildup inside the chamber; (b) place the macrocarrier and macrocarrier holder assembly, facing down, inside the chamber. [3, 8]
4. Place the PDA plate containing conidia from Materials step 6 into the chamber at a target distance of either 3 or 6 cm facing up and without lid. [9] Close chamber door.
5. Press the Vacuum switch and hold until the vacuum of about 28″ of Hg has been reached. [3]
6. Press the Fire switch and hold to allow Helium to flow from the tank and build up in chamber until the rupture disk bursts, releasing a high velocity downward pressure of Helium to collide with the macrocarrier. The microparticles coated with DNA dried on the macrocarrier disk will fly through the mesh wire of the stopping screen and penetrate the conidia sitting on the PDA plate. [3] Release the Fire switch once the rupture disk has ruptured (a popping sound can be heard).

12.3.4 Post-Transformation Procedures

Keep on working aseptically during the post-bombardment operations. The time for the transformant colonies to appear may vary from 3 days to 3 weeks depending on the fungal species.

1. Remove the plate from the chamber and incubate at 28 °C.
2. After 2 h, add 250 µL of 0.9 % w/v NaCl, 0.01 % v/v Tween 80 onto the fungal conidia in the center of the PDA plate and spread to cover the entire plate using a sterile glass spreader. Transfer plates back to 28 °C for a further 4 h.
3. After incubation, overlay each plate with 10 mL of PDA containing sufficient hygromycin B to give a final concentration of 60 µg/mL. [10]
4. Once the overlay is set, incubate plates at 28 °C until transformant colonies start appearing. [11]
5. Pick transformants and patch onto fresh PDA plates containing 60 µg/mL hygromycin B. Incubate plates at 28 °C; only colonies that survived the second selection are considered true transformants.

12.4 Notes

1. Gold microcarriers supplied by Bio-Rad range from 0.6 to 1.6 µm in diameter and tungsten beads from 0.7 to 1.7 µm in diameter. The prize of gold microparticles is about 2.5 times higher compared to the tungsten particles.
2. Rupture disks are available to accommodate Helium pressures between 450 and 2,200 psi. The choice depends on the cell type and bombardment distance.
3. Documents about the full description, operation, and the general terms and conditions regarding the use and purchase of the PDS-1000/He Particle Delivery System can be downloaded from the Bio-Rad website, URL: http://www.bio-rad.com/en-au/product/pds-1000-he-hepta-systems
4. Spread the conidia in a 2.5 cm diameter circle in the middle of the agar plate.
5. When not in use, wrap the lid of tube containing washed microparticles with parafilm to keep water from evaporation. Vortex vigorously to disperse particles before use.
6. It is important to vortex the mixture thoroughly for at least 2 min to ensure homogeneous mixing and complete spread and coverage of the particles with DNA.
7. Prepare enough stopping screens by spraying with 70 % Ethanol and leave to dry before use.
8. The flying macrocarrier disk will be blocked by the stopping screen, and only the microparticles coated with DNA will accelerate from the macrocarrier surface downward towards the target cells (e.g., fungal conidia) sitting on the agar plate.

9. The different target distances can be systematically tested for each fungus as described for *T. reesei* (Te'o et al. 2002). The three basic parameters to be tested are the vacuum, bombardment distance, and size of the microparticles.
10. Add sufficient hygromycin B to the 10 mL overlay agar taking into account that there is already 20 mL PDA agar in the plate. Final volume of PDA will be 30 mL. In order to reduce background growth, testing different antibiotic concentrations should be performed prior to transformation to determine the correct antibiotic concentration for different fungi. In addition to selection markers that render the cells resistant to a particular antibiotic, nutritional markers such as *amdS* can be used (Hazell et al. 2000). In this case, the top layer will contain acetamide as a sole nitrogen source.
11. The appearance of transformant colonies will be different between fungal species. *T. reesei* transformants when small will appear like small fibrous networks before they develop into colonies covered with conidia and have green to yellow mixture of colors.

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Transformation of Zygomycete *Mortierella alpina* Using Biolistic Particle Bombardment

13

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13.1 Introduction

Polyunsaturated fatty acids (PUFAs) play important roles not only as structural components of membrane phospholipids but also as precursors of the eicosanoids of signaling molecules, including prostaglandins, thromboxanes, and leukotrienes, which are essential for all mammals. The genus *Mortierella* has been shown to be one of the promising single cell oil (SCO) sources rich in various types of C20 PUFAs (Amano et al. 1992), after several *Mortierella* strains were reported to be potential producers of arachidonic acid in 1987 (Yamada et al. 1987). In particular, several *M. alpina* strains have been extensively studied for the production of arachidonic acid (Shinmen et al. 1989). Some of them are now used for the commercial production of SCO rich in arachidonic acid. *M. alpina* has the unique ability to synthesize a wide range of fatty acids and has several advantages not only as an industrial strain but also as a model for lipogenesis studies.

Biolistic particle bombardment is frequently used to deliver genes into intact cells. The biolistic PDS-1000/He device (Bio-Rad Laboratories, Hercules, CA, USA) delivers tungsten or gold particles coated with DNA into cells of various organisms such as bacteria (Shark et al. 1991), algae (Daniell et al. 1990), fungi (Te'o et al. 2002), and higher plants (Bruce et al. 1989). Biolistic transformation of different targets needs optimization of parameters such as vacuum pressure, target distance, helium pressure, particle type, and particle size.

The first reported transformation of *M. alpina* was through traditional protoplast-mediated transformation (Mackenzie et al. 2000). This requires optimization of protoplast formation for different types of *M. alpina*. Alternatively, the *Agrobacterium tumefaciens*-mediated transformation (AMT) technique is widely used for fungal transformation (de Groot et al. 1998; Ando et al. 2009). However, the AMT method is lengthy, including construction of *A. tumefaciens* possessing a plasmid vector and infection of target cell with *A. tumefaciens*. In this chapter, we describe the transformation of the oleaginous fungus *M. alpina* using intact spores and the biolistic particle bombardment system (Takeno et al. 2004a, b).

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13.2 Materials

1. Potato dextrose agar (PDA, BD Diagnostics, Sparks, MD, USA).
2. Czapek-Dox Broth (BD Diagnostics).
3. Tissue culture flasks (Product No. 90151, TPP Techno Plastic Products AG, Trasadingen, Switzerland).
4. Tween 80 solution prepared by sterilizing 1 L of water containing approximately 0.5 g of Tween 80 (Wako Pure Chemical Industries, Osaka, Japan).
5. Cleaning brush (see Fig. 13.1b).
6. Buchner funnel with a glass disc (60 mm diameter; rough porosity grade; 125 mL capacity) from Corning Incorporated Life Sciences (Tewksbury, MA, USA).
7. Miracloth from EMD Millipore Corporation (Billerica, MA, USA).
8. SC agar medium—6.7 g of yeast nitrogen base w/o amino acids, 20 g of glucose, 20 mg of adenine, 2 mg of histidine, 4 mg of lysine, 4 mg of tryptophan, 5 mg of threonine, 6 mg of isoleucine, 6 mg of leucine, and 6 mg of phenylalanine/liter.
9. 5-Fluoroorotic acid (5-FOA., Wako Pure Chemical Industries).
10. 2 × CTAB buffer—2 % cetyl trimethyl ammonium bromide, 0.1 M Tris/HCl(pH 8.0), 20 mM EDTA (pH 7.8), 1.9 M NaCl, and 1 % polyvinylpyrrolidone.
11. DNA. In the example pDura5 was used (Fig. 13.2, Takeno et al. 2004b).
12. GY medium—2 % glucose and 1 % yeast extract (pH 6.0).

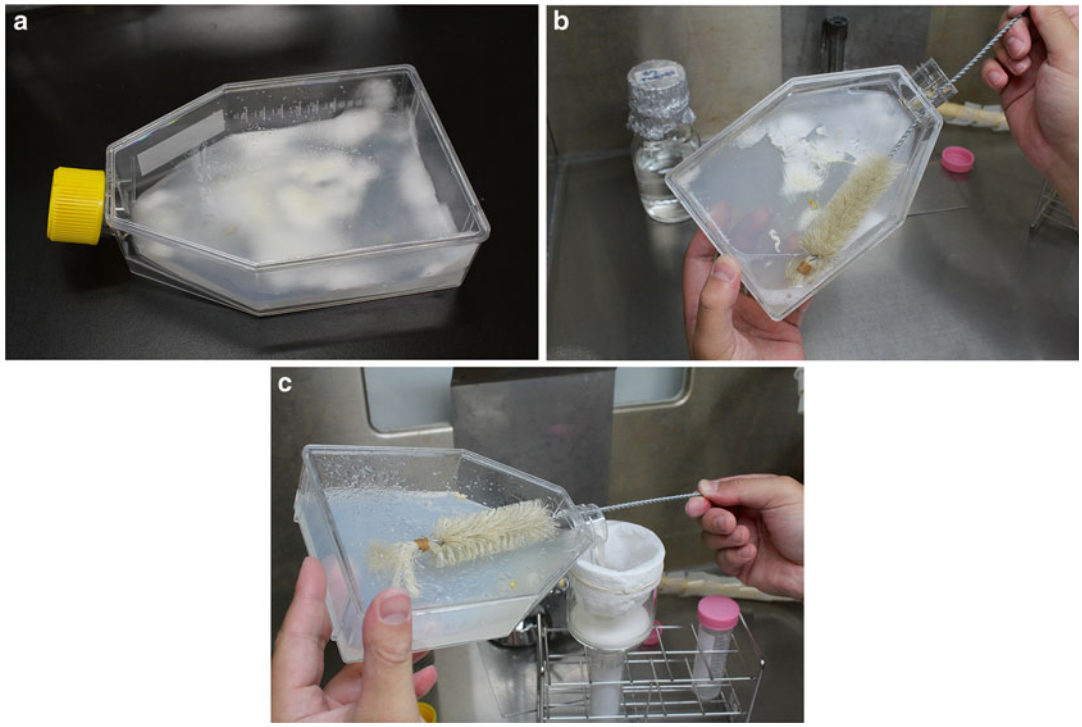

Fig. 13.1 Formation and collection of spores from *M alpina*. (**a**) Mycelia and spores on the Czapek-Dox media in a Tissue Culture Flask 150, (**b**) scraping spores from the surface of the medium, and (**c**) collection of spores using a Buchner funnel with a glass disc

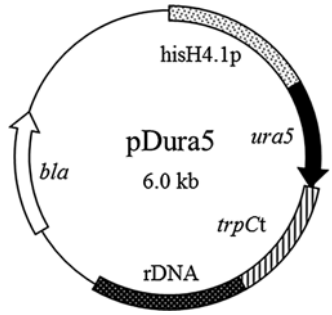

Fig. 13.2 A transformation vector, pDura5, for *M. alpina*. *bla*, ampicillin resistance gene; hisH4.1p, *M. alpina* histone H4.1 promoter; trpCt, *Aspergillus nidulans* trpC transcription terminator; rDNA, *M. alpina* 18S rDNA fragment; *ura5*, *M. alpina* orotate phosphoribosyl transferase gene

13. RNase A (Sigma, St. Louis, MO, USA).
14. TE buffer—10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA.

13.3 Methods

13.3.1 Formation of Spores from *M. alpina*

1. Inoculate mycelia of *M. alpina* on PDA and cultivate at 28 °C for more than 5 days to yield fresh mycelia as a seed for the following culture.
2. Pour 150 mL of autoclaved Czapek-Dox broth containing 2 % agar into a tissue culture flask. Inoculate the fresh mycelia onto six spots on the surface of the Czapek-Dox medium, and cultivate at 28 °C for 2 weeks, followed by at least 2 weeks at 4 °C to induce spore formation (Fig. 13.1a). This culture flask can be kept as a spore stock at 4 °C for approximately 3 years.

13.3.2 Preparation of Spore Suspensions

1. Pour 30 mL Tween 80 into the tissue culture flask and scrape off both mycelia and spores with a cleaning brush for scientific instruments (Fig. 13.1b).
2. Filter the spore suspension through a Buchner funnel with a glass disc (60 mm diameter; rough porosity grade; 125 mL capacity). The Buchner funnel is equipped with nylon gauze Miracloth. Collect the filtrate containing spores in sterilized tubes (Fig. 13.1c). Rinse the surface of the agar in the tissue flask with 30 mL Tween 80 twice and collect the remaining spores using the same Buchner funnel.
3. Centrifuge at 8,000 × *g* for 10 min and discard the supernatants.
4. Wash the spores with 50 mL of sterilized water with gentle shaking, centrifuge, and discard the supernatant.
5. Add sterilized water to adjust the spore concentration to approximately 1×10^9 spores/mL. Determine the spore number with a Burker-Turk counting chamber.

13.3.3 Isolation of Uracil Auxotrophs from *M. alpina*

1. Plate 100 μL of a spore suspension (1×10^9 spores/mL) on SC agar medium containing 1.0 mg/mL 5-FOA.
2. Incubate the dish at 28 °C for 4–7 days.
3. After isolation of uracil auxotrophs, transfer them to fresh SC agar medium without uracil and to fresh SC agar medium containing 1.0 mg/mL 5-FOA, and incubate at 28 °C for 4–7 days.
4. Check the growth rate. Uracil auxotrophs grow on SC agar medium containing 1.0 mg/mL 5-FOA, but not on SC agar medium without uracil.
5. Check mutation sites in the nucleotide sequences of orotate phosphoribosyl transferase (URA5) and orotidine-5'-phosphate decarboxylase (URA3) genes from the isolates. All mutation sites in the uracil auxotrophs isolated previously from *M. alpina* were found on the *ura5* gene, not on the *ura3* gene.

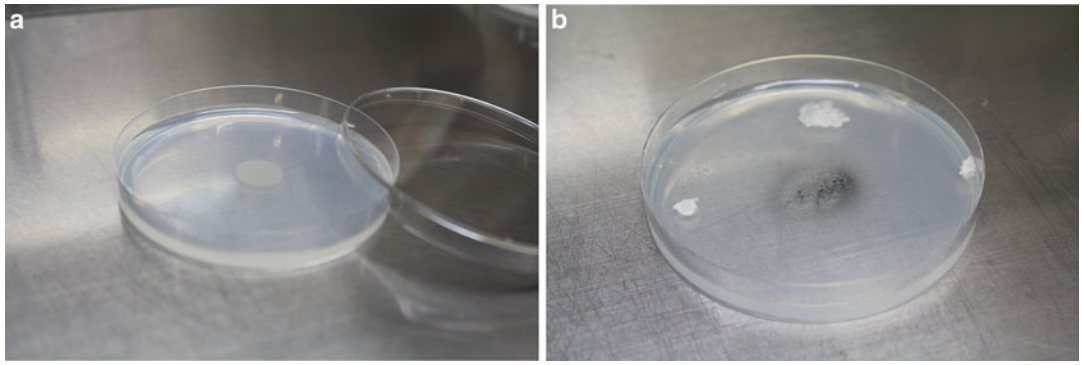

Fig. 13.3 Spore suspension placed on the medium. (**a**) Spore suspension placed at the center of the disc before biolistic particle bombardment and (**b**) transformants that appeared on the medium after biolistic particle bombardment

13.3.4 Preparation of Tungsten Particles Coated with Plasmid DNA

1. Add 30 mg of M17 tungsten particles with a diameter of 1.1 μm to a 1.5 mL microfuge tube.
2. Add 1 mL of 70 % ethanol (v/v).
3. Vortex vigorously for 3–5 min.
4. Allow the particles to soak in 70 % ethanol for 15 min.
5. Centrifuge the tube for 5 s.
6. Remove the supernatant.
7. Repeat the following wash steps (8–12) three times.
8. Add 1 mL of sterile water.
9. Vortex vigorously for 1 min.
10. Allow the particles to settle for 1 min.
11. Spin down the particles by brief centrifugation.
12. Remove the supernatant.
13. Add 500 μL of sterile 50 % glycerol and vortex vigorously.
14. Transfer 50 μL of microcarriers to a new microcentrifuge tube.
15. Add 5 μL of plasmid DNA pDura5 (1–5 μg/μL), 50 μL of 2.5 M $CaCl_2$, and 20 μL of 0.1 M spermidine in that order, while vortexing vigorously in between.
16. Continue vortexing for 2–3 min.
17. Allow the microcarriers to settle for 1 min.
18. Centrifuge the tube for 2 s.
19. Remove the supernatant.
20. Add 140 μL of 100 % ethanol.
21. Remove the supernatant.
22. Add 48 μL of 100 % ethanol.
23. Gently resuspend the pellet by tapping.
24. Load 8 μL of microcarriers onto a macrocarrier set on a macrocarrier holder.

13.3.5 Transformation of *M. alpina* with the Biolistic Particle Bombardment System

1. Place 100 μL of a spore suspension (1×10^9 spores/mL) at the center of a Petri dish (90 mm diameter) containing SC agar medium without uracil. Do not spread the suspension over the whole surface of the medium. The area of the spore suspension should be small enough to be a good target for the biolistic particles.
2. Dry the spore suspension by placing the dish for 30–60 min on a clean bench with the lid off (Fig. 13.3a).
3. Set the conditions for the biolistic particle bombardment system as follows: Vacuum, 28 in. Hg; target distance, 6 cm; He pressure, 1,100 psi; tungsten particle size, 1.1 μm.
4. Perform the bombardment twice under the same conditions. (Two bombardments frequently allow us to obtain more transformants. It might enhance the delivery rate of tungsten particles into spores.)

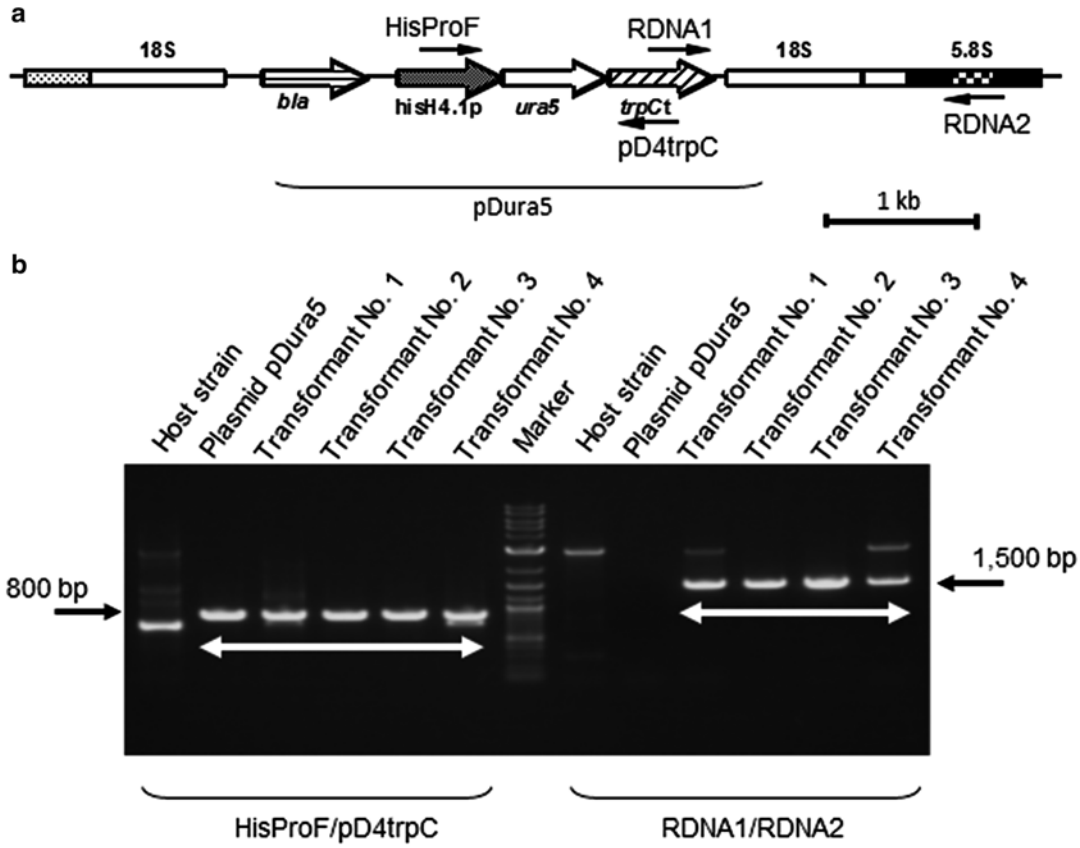

Fig. 13.4 Characterization of transformants through PCR. (**a**) Schematic representation of pDura5 integrated at the rDNA locus, and (**b**) confirmation through agarose gel electrophoresis

5. Add 70 µL of sterilized water to the dish and spread the bombarded spores over the whole surface of the medium.
6. Incubate at 28 °C for 4–7 days and isolate transformants on SC agar medium without uracil until transformants appear (Fig. 13.3b).

13.3.6 Isolation of Genomic DNA from *M. alpina* Transformants

1. Cultivate *M. alpina* in 50 mL GY for 5 days at 28 °C with shaking.
2. Collect the mycelia by filtration.
3. Freeze 2–5 g (wet mass) mycelia in liquid nitrogen and grind with a mortar.
4. Ground mycelia in 20–30 mL 2 × CTAB buffer and transfer to a sterile tube.
5. Incubate for 20–30 min at 65 °C.
6. Let the mycelia settle at room temperature for 20 min.
7. Extract genomic DNA from the lysate with phenol/chloroform (1:1; v/v), precipitation with isopropanol, treatment with RNaseA, and re-precipitation with ethanol.
8. Dissolve the genomic DNA in 1 mL of TE buffer.

13.3.7 Characterization of Transformants by Genetic Analysis

1. Perform PCR to confirm the genetic makeup of the transformants. Confirmation of transformed cells is done through PCR with primers RDNA1 and RDNA2 (Fig. 13.4a). These primers will only amplify a PCR

product when pDura5 is integrated correctly into the chromosomal rDNA locus. In addition, vector-specific reverse (pD4trpC) and forward primers (HisProF) are used to confirm whether the transformation has been successful at all (Fig. 13.4a). Gene fragments of appropriate sizes are detected only in correct transformants (Fig. 13.4b).

2. The nucleotide sequences of amplified products can be determined to confirm correctness.

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Part V

Transformation Methods: Agrobacterium-Mediated Transformation

Agrobacterium tumefaciens-Mediated Transformation

14

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14.1 Introduction to *Agrobacterium tumefaciens*-Mediated Transformation

The Gram negative plant pathogenic bacterium *Agrobacterium tumefaciens* relies on genetic transformation of its host plant, resulting in tumorous growth of transformed cells and a subsequent dramatic change in their metabolism. During the initial stages of the infection, the bacterium transfers part of its genome found on a >200 kb large tumor-inducing plasmid (Ti). The transferred DNA (T-DNA) is bordered by two directional imperfect repeats (called left and right border) and contains genes that encode for enzymes responsible for the formation of plant hormones, which cause tumors growth, and the formation of metabolites which only the bacterium can degrade. In addition to the T-DNA region, the Ti-plasmid also encodes the T-DNA transfer machinery (virulence genes) that is responsible for recognizing the presence of a plant host (based on phenolic compounds), enzymes for liberating the T-DNA region from the Ti-plasmid and structural proteins for the formation of a transfer tube between the bacterium and the host cells (Citovsky et al. 2007). The T-DNA region is liberated from the Ti-plasmid as single-stranded DNA that is coated with VirE2 and VirD2 proteins. The coating prevents the T-DNA from forming secondary structure, protects it from degradation during the transfer process and targets the DNA to the nucleus of the host plant (Zupan et al. 2000).

This natural genetic transformation system was first utilized by the plant research community to transform various model plant species, and was termed *Agrobacterium tumefaciens*-mediated transformation (AMT) (Schell and Van Montagu 1977). The finding that the T-DNA transfer machinery was able to act *in trans* on T-DNA not located on the Ti-plasmid allowed for the development of binary vector systems. In these, the T-DNA on the Ti-plasmid has been moved to small shuttle plasmids, which are easier to manipulate by standard molecular biological techniques. In these plasmids the tumour causing genes have been eliminated from the T-DNA, giving room for a large amount of genetic cargo (Hoekema et al. 1983).

The first use of the AMT technique on a fungus was described in 1995 by Bundock et al. working with *Saccharomyces cerevisiae* (Bundock et al. 1995). A few years later, de Groot and co-workers showed that the method was also applicable to several ascomycete species and a single basidiomycete (de Groot et al. 1998). Up to date, over 130 different fungal species, including

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ascomycetes, basidiomycetes, oomycetes, and zygomycetes have successfully been transformed using the AMT technique (Frandsen 2011). The great advantage with AMT in comparison to most other available systems for genetic transformation of fungi is that it is independent of the formation of protoplasts, and typically only results in a single T-DNA insert per transformant (de Groot et al. 1998). The high rate of transformants with a single insert makes the technique ideal for forward genetic studies aiming at identifying genes which affect a given phenotype, by screening large libraries of random mutants, as it increases the likelihood that only a single gene is mutated in the individual transformants (Li et al. 2007). Random integration of the T-DNA has in several studies, with different species, been shown to display a bias towards transcribed genes, promoters, and the 5' end of genes (Choi et al. 2007). Initially this bias might seem undesirable, however, it efficiently reduce the size of the library one has to generate to obtain a saturated mutant library for transcribed protein encoding genes. In connection with targeted genome modification experiments, e.g., gene replacement, AMT typically result in a three to sixfold increase in gene targeting efficiency compared to $CaCl_2$/PEG-protoplast-based transformation, as shown for *Aspergillus awamori* (Michielse et al. 2005). The higher targeting frequency has been hypothesized to be due to the single-stranded nature of the T-DNA and its VirE2/VirD2 coat which might promote homologous recombination and thereby targeted integration (Michielse et al. 2005).

Since the pioneering work of Bundock et al. and de Groot et al. only few ground-breaking advances has been made and the basic AMT protocol for fungi remains largely unchanged, likely due to the fact that the technology was already matured when it was adopted from the plant research community. One area where progress has been made is AMT of basidiomyces. While AMT of ascomycete species was very successful from the first use of AMT of fungi, its application to basidiomyces was troublesome and impossible for the majority of species tested; only few have reported their failures. However, in 1999 Lugones and co-workers showed that the lack of success was due to the incompatibility of the promoters used to drive the expression of the used selection marker gene and the basidiomyces transcription/translation machinery (Lugones et al. 1999). Engineering of the selection marker cassettes by replacing the previous used ascomycete promoter with promoters from basidiomyces, such as *Agricus bisporus* and *Coprinopsis cinerea*, solved the problem (Burns et al. 2006 and McClelland et al 2005). Though these advances have been driven by experiments relying on AMT, they have revealed that the main problem with transforming basidiomyces was not the transfer and integration of DNA but the used selection marker cassettes.

Compared to other transformation techniques AMT also allows for the transfer of very large DNA fragments, Takken and co-workers documented the transfer of up to 75 kb DNA fragments into *Fusarium oxysporum* and *A. awamori* (Takken et al. 2004). Construction of the large T-DNA bearing plasmids for these experiments was based on the conversion of bacterial artificial chromosome (BAC) by the introduction of a fungal selection marker gene and the two T-DNA borders in inverse orientation, resulting in a binary BAC (BIBAC). A technique initially developed in the plant research community for complementation screening where it has been used to transfer up to 350 kb (Hamilton et al. 1996).

Another technique originally developed by the plant research community, which is now being exploited by fungal researchers, is the simultaneous introduction of multiple different T-DNA regions into the same host cell, by co-culturing the recipient organism with multiple different *A. tumefaciens* strains. A principle that was used by Wang et al. to increase the gene targeting efficiency in *Grosmannia clavigera*, by introducing two different T-DNAs each containing two thirds of the selection marker (known as split-marker/bipartial marker strategy) (Wang et al. 2010).

The main general advances within the AMT field have been made in the way binary vectors compatible with AMT are constructed, including the development of new selection marker

cassettes and single step construction of binary plasmids for targeted genome modification. In the following sections one of these are described in details, however please note that many others exists, each offering different advantages and levels of compatibility with up- and downstream experiment steps (Frandsen 2011).

14.2 User-Bricks: A New Strategy for Constructing Binary Plasmids

The Uracil Specific Excisions Reagent (USER) cloning technique allows for efficient ligation-free directional cloning of PCR amplified DNA fragments into vectors which contain a USER cloning site (UCS). Key to the technique is the recognition and removal of 2-deoxyuridine bases found in the primer regions at either end of the PCR amplicon, resulting in the formation of long (typically 9–12 bp) 3' overhangs. The two 2-deoxyuridine bases are introduced into the ends of the PCR amplicon as part of two unique 5' primer overhangs that are identical to sequences found in the UCS of the recipient plasmid. Compatible 3' overhangs on the plasmid are prepared by the combined digestion of the UCS with a standard and a nicking Type II endonuclease (Nour-Eldin et al. 2006). A variant of the USER cloning technique, known as USER Fusion, relies entirely on PCR amplified DNA fragments (Geu-Flores et al. 2007). This technique has primarily become possible due to the development of proofreading DNA polymerases that do not stall at the 2-deoxyuridines in the primers, which has significantly reduced the risk of PCR introduced mutations (Nørholm 2010).

The USER Fusion technique has allowed my lab to develop a new cloning strategy (USER-Bricks) for fast and efficient construction of vectors intended for performing targeted genome modifications (Sørensen et al. 2014). The USER-Brick system consists of a set of standardized building blocks, e.g., vector backbones, selection markers, and promoters, that can be combined to generate vectors intended for gene replacement, *in locus* overexpression, GFP-tagging, and ectopic expression. The modular layout allows for easy experimental design and in-laboratory construction, as stock of the various building blocks can be generated, subjected to quality control, and stored until needed. The incentive for developing the new system has been a desire to eliminate the need for time consuming and often inefficient restriction enzyme digestion of the recipient USER plasmids (Frandsen et al. 2008). Many have experienced problems with incomplete digestion and nicking of the recipient vectors, resulting in a reduced number of transformants and a significant reduction in correctly assembled plasmids. In this new system, PCR is used to amplify the vector backbone using primers that generate unique single-stranded overhangs after treatment with the USER enzyme mix. This strategy eliminates false positive transformants caused by undigested plasmids, leaving only the false positives that are the result of primer synthesis errors. The system has been used to join up to ten DNA fragments in a single cloning reaction, with an efficiency of approximately 95 %.

The overhangs on the different building blocks are unique, and thus allows for directional assembly of the fragments in a single cloning reaction. The vector backbone is amplified as two individual fragments each containing half of the kanamycin resistance gene, a strategy that ensures that only correctly assembled plasmids will result in resistant *E. coli* transformants. Currently the system consists of two different vector backbones, pAg1 which has previously been used for USER cloning and pPK2 which has been the most popular backbone in fungal laboratories relying on AMT (Frandsen 2011). These backbones can be combined with three different fungal selection marker cassettes (promoter::gene::terminator), conferring resistance to hygromycin, geneticin, and DL-phosphinothricin (BASTA), respectively. New marker cassettes can easily be introduced into the system by designing primers for amplifying the new cassette and adding appropriate USER cloning 5' overhangs on the primers, as described in the section "Primers for amplifying the generic USER-Bricks."

14.3 Different Types of AMT Experiments

14.3.1 Experiments Relying on Random Integration into the Genome

For random mutagenesis purposes, which depends on the "non-homologous end joining" (NHEJ) DNA repair pathway, any AMT compatible vector with an appropriate fungal selection marker gene can be used in combination with the described generic AMT protocol.

For ectopic expression of a given gene, the USER-Brick system allows for cloning of the gene with its native regulatory elements (promoter and terminator) (Fig. 14.1) or with an exogenous promoter element (Fig. 14.2). The system currently features two different exogenous promoters, *PgpdA* and *PalcA*. The *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase promoter (*PgpdA*) provides high constitutive expression of the gene it regulates. This promoter has successfully been utilized in numerous experiments with many different fungal species, as it is used to drive expression of fungicide resistance genes in many vectors. The *A. nidulans* alcohol dehydrogenase promoter (*PalcA*) allows for tight regulation of gene expression in *Aspergillus* species (Waring et al. 1989). The gene to be expressed should be amplified by PCR with primers that targets the entire codon sequence (gene-specific primers = GSP), ranging from the G of the start codon to the stop codon, plus 500 bp downstream to ensure that the terminator region is included. The addition of 5' USER cloning overhangs to the GSP primers will result in the formation of the necessary start codon in the forward primer, as described in section "Primer design for the gene of interest."

14.3.2 Experiments Relying on Targeted Integration into the Genome

Targeted mutagenesis experiments rely on the homologous recombination (crossover events) DNA repair pathway for integration of the introduced T-DNA into the genome of the recipient

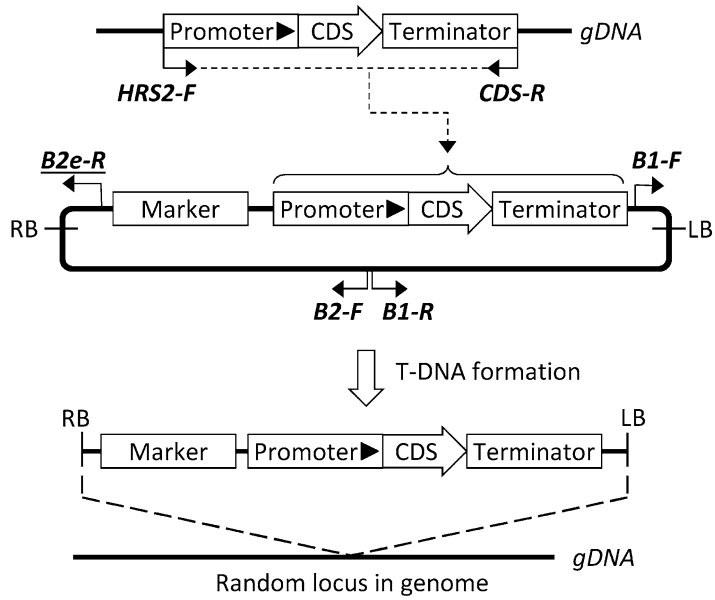

Fig. 14.1 Ectopic expression of a gene with its native regulatory elements. Primers are represented by *solid arrows*. *CDS* coding sequence of gene to be expressed, *Term* terminator region of gene to be expressed, *gDNA* genomic DNA, *RB* and *LB* right and left border repeats, *T-DNA* transfer DNA region. Note the use of the B2e-R for amplification of the backbone to allow for directly joining with the marker cassette (any of the three available dominant markers). Alternatively use the B3 + B4 backbone elements

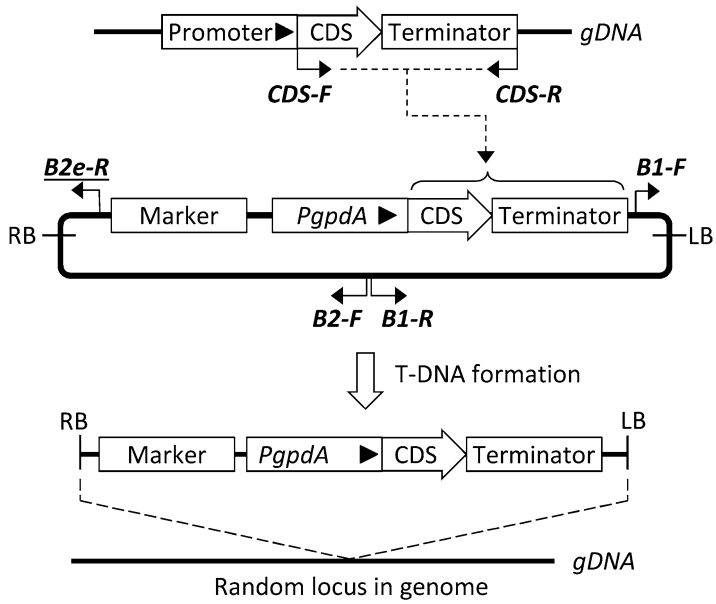

Fig. 14.2 Ectopic expression of a gene controlled by one of the two exogenous promoters. Primers are represented by *solid arrows*. *CDS* coding sequence of gene to be expressed, *Term* terminator region of gene to be expressed, gDNA genomic DNA, *RB* and *LB* right and left border repeats, *T-DNA* transfer DNA region. Select between *PgpdA* or *AlcA* promoter. Alternatively use the B3+B4 backbone elements or any of the three dominant selection markers. Note the use of the B2e-R for amplification of the backbone to allow for directly joining with the marker cassette

organism (van Attikum et al 2001). The frequency of homologous recombination (HR) varies considerably between fungal species, ranging from almost 100 % in *Saccharomyces cerevisiae* (baker's yeast) to 0.04 % in *Blastomyces dermatitidis* (Gauthier et al. 2010). The large variation is due to differences in the activity of the NHEJ and HR DNA repair pathways in the individual species, resulting in either of the two out-competing the other. Low HR frequency in fungal species can be improved by disrupting the NHEJ pathway, which is typically achieved by replacing/inactivating one of the genes in the NHEJ pathway, generally the *Ku70* gene (van Attikum et al 2001). The HR frequency is in addition dependent on the length of the used homologous recombination sequences (one located upstream and one located downstream of the targeted gene), where an increase in length typically has a positive effect on the HR frequency.

Targeted replacement (deletion) of a gene via HR requires the amplification of two homologous recombination sequences (Fig. 14.3). For *in locus* expression (exchange of the natural promoter) the upstream primer pair designed for the replacement experiment can be reused, while the second downstream primer pair should be designed to amplify the start of the target gene, from the G in the start codon (Fig. 14.4). As in the other expression experiments, addition of the appropriate 5' USER overhangs will create the required start codon in the forward primer. The placement of the reverse primer should be app. 1,500 bp from the start of the gene. For heterologous expression from a predetermined locus in the genome (Fig. 14.5), three primer pairs are needed. One pair for the gene to be expressed, located as described for the situation in Fig. 14.1, and two pairs for the homologous recombination sequences surrounding the target locus, as described for Fig. 14.3. To allow for fusion of the expression cassette (promoter-CDS-terminator) with the downstream homologous recombination sequence (HRS2), it is necessary to use the alternative primer overhangs CDSf-R and HRSf2-F to create compatible overhangs.

The USER-Brick system also allows for the construction of transcriptional reporters that can be used to monitor in situ expression. The reporter system can either be integrated randomly into the genome (Fig. 14.6) or at a fixed locus (Fig. 14.7). In both cases the

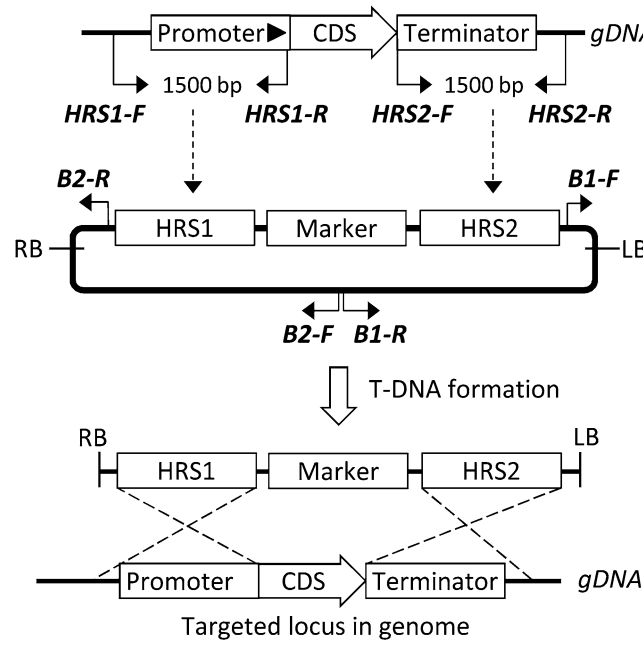

Fig. 14.3 Targeted gene replacement. Alternatively use the B3 + B4 backbone elements and any of the three dominant selection markers. Primers are represented by *solid arrows*. *CDS* coding sequence of gene to be expressed, *gDNA* genomic DNA, *RB* and *LB* right and left border repeats, *HRS1* and *HRS2* up- and downstream homologous recombination sequence, specific for target locus, *T-DNA* transfer DNA region

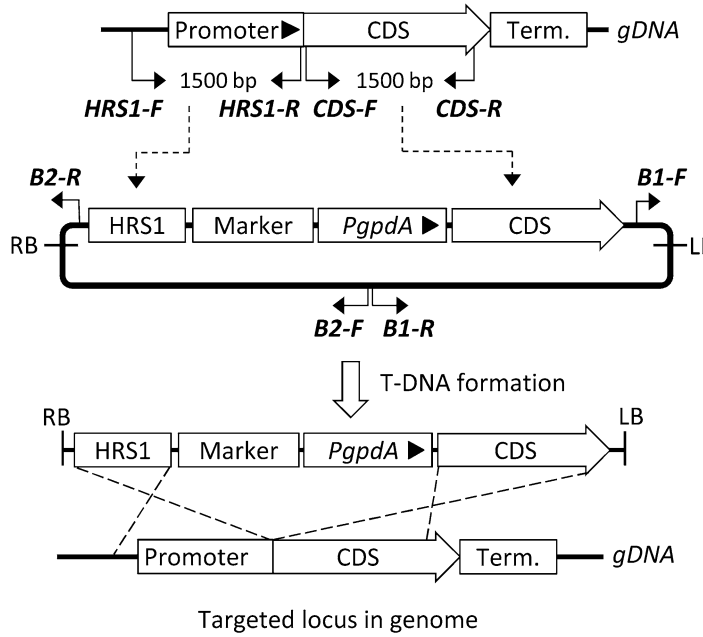

Fig. 14.4 *In locus* overexpression experiments (promoter exchange). Primers are represented by *solid arrows*. *HRS1* and *HRS2* up- and downstream homologous recombination sequence, specific for target locus, *CDS* coding sequence of gene to be replaced, *gDNA* genomic DNA, *RB* and *LB* right and left border repeats, *T-DNA* transfer DNA region. Alternatively use the B3 + B4 backbone elements, any of the three dominant selection markers and either of the two available promoters (*PgpdA* or *PalcA*)

fluorescent reporter, e.g., monomeric red fluorescent protein (mRFP), is PCR amplified and fused with the promoter one wants to analyze. Note that it is necessary to use different reverse primers for amplifying the mRFP depending on whether one wants to use the random or targeted integration strategies, mRFP-R and mRFPf-R, respectively.

14.4 Guide for Optimizing AMT in a New Species: Which Parameters to Focus on?

The transformation frequency when performing AMT has been shown to be affected by many different parameters, such as incubation temperature

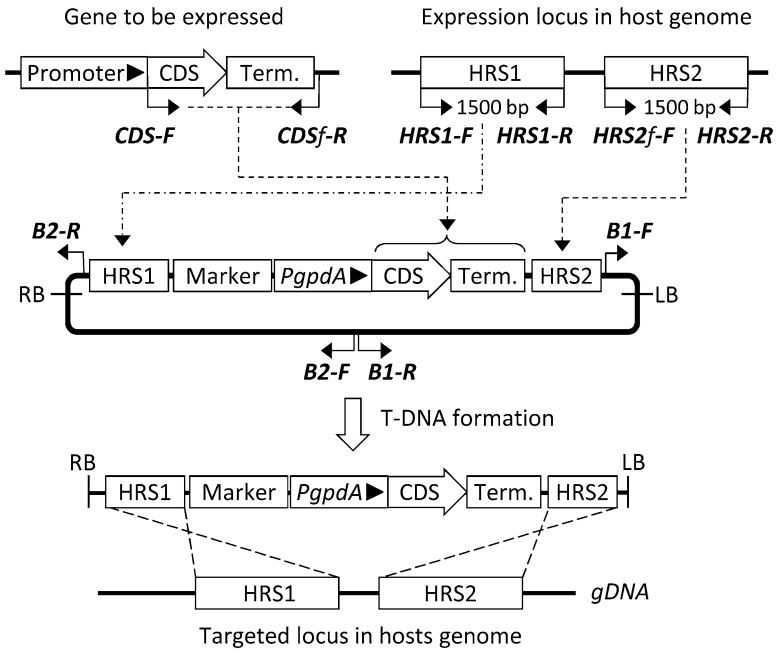

Fig. 14.5 Heterologous expression from a fixed locus in the genome. Primers are represented by *solid arrows*. *CDS* coding sequence of gene to be expressed, Term terminator region of gene to be expressed, *HRS1* and *HRS2* up- and downstream homologous recombination sequence, specific for target locus, *gDNA* genomic DNA, *RB* and *LB* right and left border repeats, *T-DNA* transfer DNA region. Alternatively use the B3 + B4 backbone elements, any of the three dominant selection markers and either of the two available promoters. Note the use of the CDSf-R and HRS2f-F overhangs to allow for fusion of CDS and HRS2

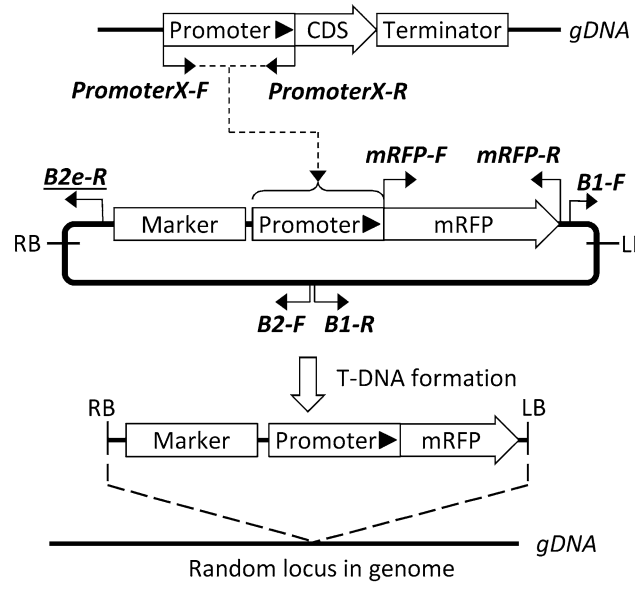

Fig. 14.6 Random integration of transcriptional reporter system. Primers are represented by *solid arrows*. *Promoter*(*X*) promoter to be tested, *mRFP* monomeric red fluorescent protein, *gDNA* genomic DNA, *RB* and *LB* right and left border repeats, *T-DNA* transfer DNA region. Note the use of the B2e-R for amplification of the backbone to allow for directly joining with the marker cassette (any of the three available dominant markers). Alternatively use the B3 + B4 backbone elements

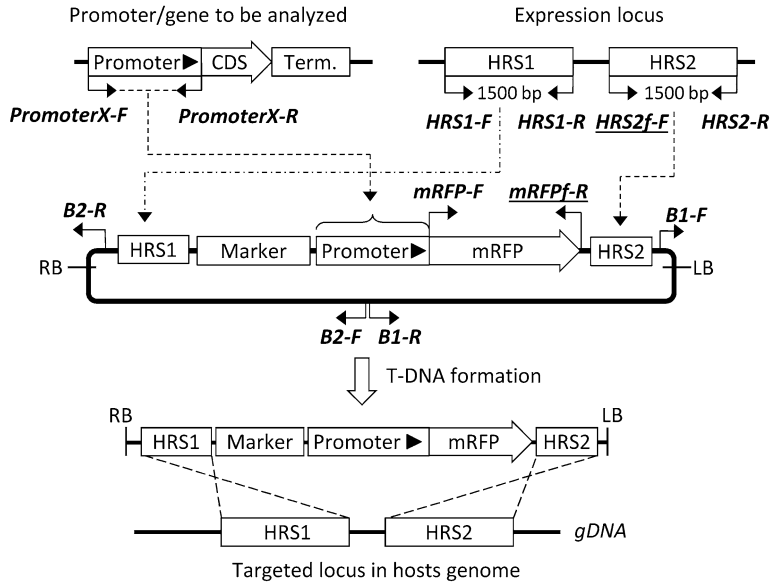

Fig. 14.7 Targeted integration of transcriptional reporter system. Primers are represented by *solid arrows*. *PromoterX* promoter to be tested, *mRFP* monomeric red fluorescent protein, *HRS1* and *HRS2* up- and downstream homologous recombination sequence, specific for target locus, *gDNA* genomic DNA, *RB* and *LB* right and left border repeats, *T-DNA* transfer DNA region. Note the use of the mRFPf-R primer for amplifying the fluorescent reporter gene and HRS2f-F primer for amplifying the second targeting sequence. Alternatively use the B3 + B4 backbone elements and any of the three selection markers

and time, *A. tumefaciens* strain, plasmid backbone, fungal inoculum concentration, *A. tumefaciens* growth stage and number, preincubation prior to cocultivation, media, pH, and concentration of the inducer acetosyringone (Ando et al. 2009). Unfortunately, the optimal conditions are unique for each individual fungal species and isolates, making an optimization process advisable to obtain the highest possible transformation frequency.

When optimizing the AMT protocol for a fungal species that has not previously been transformed by this technique, it is advisable to start by testing the sensitivity of the fungus to the fungicide to be used and determine a minimal concentration for efficient growth inhibition. The most reliable results are obtained by simulating the AMT process including the initial cocultivation step, where the fungal spores/fragments germinate and grow on filters on induction media with acetosyringone (IMAS) without selective pressure, and then transferring the filters with cells to the selective medium. The selective pressure should be high enough to inhibit growth of non-transformed fungal cells which will ease the identification of transformants and minimize the risk of isolating false positives.

The next parameter to optimize is the temperature during the cocultivation and the duration of this step. Prolonged cocultivation typically leads to an increased number of transformants; however, the rate of multiple T-DNA integration events in single transformants also increases due to multiple transfer events (Combier et al. 2003). A situation that is not desirable if the aim is targeted genome modifications or random mutagenesis, but which can be an advantage in experiments aimed at production of high levels of a given protein or metabolite. The optimal temperature during cocultivation is typically between 20 and 25 °C, which is best explained by the finding that the T-DNA transfer machinery is inactivated at temperatures above 28 °C during AMT (Fullner and Nester 1996).

The combination of *A. tumefaciens* strain and binary plasmids used can also affect the transformation frequency significantly (Yamada et al 2009; Wei et al. 2010); however, no single combination has been proven optimal for a larger number of fungal species.

If necessary, also the amount of fungal inoculum and the density of the *A. tumefaciens* culture used for the cocultivation step should be optimized. Generally, *A. tumefaciens* cultures with an

optical density (OD_{600}) between 0.4 and 0.6 give the highest transformation frequency.

14.5 Materials

14.5.1 Equipment

- Agarose gel electrophoresis system (for analysis of DNA)
- Black filter paper (AGF 220 85 mm, Frisenette ApS, Denmark) or other filter paper or nitrocellulose membrane
- Bottle-top 0.2 µm filters for sterilization of solutions
- Centrifuge with a capacity to process up to 400 mL solution at 4,000 g and 4–25 °C
- Drigalski spatula
- Electroporation cuvette (0.2 mm electrode gap) (Bio-Rad)
- Electroporation apparatus (Bio-Rad Gene Pulser II or similar)
- 50 mL, 300 mL, 1 L Erlenmeyer flasks
- Equipment for counting spores
- 25 °C, 28 °C and 37 °C incubators with orbital shake
- Heating block/water bath,
- Miracloth (EMD Chemicals)
- PCR thermocycler
- 9 and 5.5 cm plastic Petri dishes
- Spectrophotometer and cuvettes
- Sterile toothpicks
- Benchtop centrifuge for 1.5 mL tubes
- Tubes: 1.5 mL, 15 mL and 50 mL centrifuge tubes

14.5.2 Solutions

- 10 mM Acetosyringone (AS) (CAS: 2478-38-8): Dissolve 19.62 mg AS in 10 mL sterile MilliQ-water. Stir for 1 h. Adjust the pH to 8 with 5 M KOH. Filter-sterilize and store at −20 °C. Alternatively acetosyringone can also be dissolved in DMSO or 96 % ethanol.
- Glycerol (CAS: 56-81-5) 10 % and 20 % v/v in MilliQ-water (sterile).
- D-(+)-glucose (CAS: 50-99-7) 20 % w/v in MilliQ-water (sterile).
- 1 M MES (CAS: 145224-94-8): 19.52 g MES dissolved in 80 mL MilliQ-water, adjust pH to 5.3 with 5 M KOH, and then bring the volume to 100 mL. Filter-sterilize and store at −20 °C.
- MilliQ-water or distilled water (sterile).
- 10:1 TE buffer (Tris–HCl 10 mM and EDTA 1 mM, pH 8).
- Water agar for IMAS plates: 146 mL MilliQ-water + 10 g BD Bacto Agar in a 500 mL bottle. Autoclave and remelt in microwave oven before use.

14.5.3 Enzymes and Molecular Biological Kits

- PCR purification kit (GFX PCR and gel purification kit or similar).
- PfuX7 DNA polymerase (an *E. coli* strain expressing this enzyme can be obtained free of charge) (Nørholm 2010) and commercial alternative is PfuTurbo Cx Hotstart DNA polymerase (Agilent).
- Plasmid purification kit (Qiagen Miniprep kit or similar).
- USER cloning enzyme mix (New England Biolabs).

14.5.4 Antibiotic Stocks (Hazardous and Toxic Compounds. Wear Gloves and Work in a Fume Hood)

All stock solutions are sterilized by filtration (0.22 µm filter) and stored at −20 °C in 1–2 mL aliquots.

1. Cefoxitin sodium (Cef) (CAS: 33564-30-6) 50 mg/mL stock in MilliQ water and for experiments in a concentration of 300 µg/mL.
2. Hygromycin B (HygB) (CAS: 31282-04-9) 100 mg/mL stock in MilliQ-water. For experiments with *Fusarium* species use a concentration of 150 µg/mL during the initial selection step and 100 µg/mL for subsequent steps.
3. Geneticin (G418, G-418) (CAS: 108321-42-2) 50 mg/mL stock in MilliQ-water and for experiments in a concentration of 100 µg/mL for *Fusarium* species.

4. Kanamycin sulfate (Kan) (CAS: 25389-94-0) 10 mg/mL stock in MilliQ-water and for experiments in a concentration of 50 µg/mL.
5. DL-phosphinothricin (BASTA) (CAS: 77182-82-2) 100 mg/mL stock in MilliQ-water and for experiments in a concentration of 600 µg/mL for *Fusarium* species.
6. Rifampicin (Rif) (CAS: 13292-46-1) 50 mg/mL stock in DMSO and for experiments in a 10 µg/mL.

14.5.5 Organisms and Cells

- Target organism, here *Fusarium graminearum* is used as a model organism.
- Chemical competent *E. coli* cells (DH5a, JM109 or similar).
- Electro competent *Agrobacterium tumefaciens* LBA4404 (other strains will also work).

14.5.6 Media

- Solid and liquid LB medium
- Liquid SOC medium
- IMAS-medium (solid) pH between 5 and 5.3 300 mL

2.5× Salt solution (pre-heated to 60 °C)	120.0 mL
20 % Glucose (w/v)	2.7 mL
20 % Glycerol (v/v)	7.5 mL
Water agar (melted)	145.8 mL
Cool the solution to 55 °C before adding	
MES-solution (1 M)	12.0 mL
Acetosyringone (10 mM)	6.0 mL

- *2.5× Salt solution* (*1,000 mL*):

K_2HPO_4 (CAS: 88-57-1)	5.125 g
KH_2PO_4 (CAS: 7778-77-0)	3.625 g
$(NH_4)_2SO_4$ (CAS: 7783-20-2)	1.250 g
$MgSO_4 \cdot 6H_2O$ (CAS: 30-18-1)	1.160 g
NaCl (CAS: 7647-14-5)	0.375 g
$CaCl_2 \cdot 2H_2O$ (CAS: 10035-04-8)	0.165 g
$FeSO_4 \cdot 7H_2O$ (CAS: 7782-63-0)	0.0062 g

Dissolve each salt one at a time to avoid the formation of insoluble complexes.

Filter-sterilize and store at room temperature.

Note: If clear crystals form during storage heat the solution to 60 °C for 30 min.

- Defined *Fusarium* Medium (DFM) or other appropriate media 500 mL.

D-(+)-Glucose (CAS: 50-99-7)	625 mg
L-Asparagine (CAS: 70-47-3)	661 mg
$MgSO_4 \cdot 6H_2O$ (CAS: 30-18-1)	259 mg
KH_2PO_4 (CAS: 88-57-1)	762 mg
KCl (CAS: 7447-40-7)	373 mg
1000×Trace elements	0.5 mL
BD Bacto Agar	10 g
MilliQ-water to Autoclave	500 mL

1000× Trace element solution for DFM medium (*500 mL*).

Add the salts in the listed order, and allow each to dissolve completely before adding the next.

$Na_2B_4O_7 \cdot 10H_2O$ (CAS: 1303-96-4)	20 mg
$CuSO_4 \cdot 5H_2O$ (CAS: 7758-99-8)	200 mg
$MnSO_4 \cdot 1H_2O$ (CAS: 10034-96-5)	350 mg
$Na_2MoO_4 \cdot 2H_2O$ (CAS: 10102-40-6)	400 mg
$ZnSO_4 \cdot 7H_2O$ (CAS: 7446-20-0)	5 g
$FeSO_4 \cdot 7H_2O$ (CAS: 7782-63-0)	600 mg

MilliQ-water to 500 mL and sterilize by filtration.

14.5.7 Primers

14.5.7.1 Primers for Amplifying the Generic USER-Bricks

The standard USER-Bricks required for the different types of experiments can be amplified with the primers listed in Table 14.1. The primer pairs have been designed to amplify their targets using an annealing temperature of 60 °C. New marker cassettes can be introduced into the system by designing primers for amplifying the desired resistance gene cassette, including the promoter and terminator, and then adding the appropriate USER Fusion 5' overhangs to the primers (forward primer: 5'- ACGCAATACU, reverse primer: 5'- ACTAGGTCAU). The Us in the primer are 2-deoxyuridine (the DNA analog of uracil).

Table 14.1 Primers for amplifying the different USER-Bricks

Purpose	Name:	Sequence (5' to 3')	Overhangs compatible with	PCR template	PCR Product (bp)
Backbone elements based on pAg1-H3/pRF-HU2 plasmids					
Generic	B1-F	AAGGTTTAAUTCACTGGCCGTCGTTTTA	HRS2-R	[a]	2,165
Generic	B1-R	ATTTAAAGAUCCGCGCGAGC	B2-F	[a]	
Generic	B2-F	ATCTTTAAAUGGAGTGTCTTCTTCCCA	B1-R	[a]	1,658
Generic	B2-R	AATACGACCUTCGTGACTCCCTTAATTCT	HRS1-F	[a]	
Ectopic express	B2e-R	AGTATTGCGUTCGTGACTCCCTTAATTCT	Marker-R	[a]	1,658
Backbone elements based on ppPK2 or pPZP-201BK					
Generic	B3-F	AAGGTTTAAUCGTTTCCCGCCTTCAGTTTAAA	HRS2-R	[b]	3,096
Generic	B3-R	AGCGGCTAAUCAAGGCTTCACCC	B2-F	[b]	
Generic	B4-F	ATTAGCCGCUACAAGATCGTAAAGA	B1-R	[b]	2,937
Generic	B4-R	AATACGACCUTGTGTTATTAAGTTGTCTAAGCGTCA	HRS1-F	[b]	
Ectopic express	B4e-R	AGTATTGCGUTGTGTTATTAAGTTGTCTAAGCGTCA	Marker-R	[b]	2,937
Markers					
Hygromycin marker (*PtrpC*:*hph*:*TtrpC*)	HygR1-F	ACGCAATACUAGTCGGGGGATCCTCTAG	B2-R, B4-R	[a, c]	2,480
	HygR1-R	ACTAGGTCAUGGGCCCATCGATGATCAG	HRS2-F, CDS-R, promoter-F		
Hygromycin marker (*PgpdA*:*hph*:*TtrpC*)	HygR2-F	ACGCAATACUATCTTTCGACACTGAAATACGT	B2-R, B4-R	[d]	3,989
	HygR2-R	ACTAGGTCAUTCGAGTGGAGATGTGGAGTGGG	HRS2-F, CDS-R, promoter-F		
Geneticin marker (*PgpdA-NPTII-NcTtubC*)	GenR-F	ACGCAATACUCATGCAACATGCATGTACTGTCTGAT	B2-R, B4-R	[e]	1,440
	GenR-R	ACTAGGTCAUCGCGGCTTCGAATCGTGG	HRS2-F, CDS-R, promoter-F		
Bar resistance (*PtrpC*:*barR*:*TtrpC*)	Bar-F	ACGCAATACUAAGAAGGATTACCTCTAAACAAGTG	B2-R, B4-R	[f]	1,677
	Bar-R	ACTAGGTCAUTCGACAGAAGATGATATTGAAGGAGC	HRS2-F, CDS-R, promoter-F		
Promoter					
Constitutive	PgpdA-F	ATGACCTAGUGCCAGCCCGAATTCCCTTGTATC	Marker-R	[g]	2,320
	PgpdA-R	ATACAGCCUGGGTGATGTCTGCTCAAGCGGGG	CDS-F		
Inducible	PalcA-F	ATGACCTAGUCTCCCCGATGACATACAGGAGG	Marker-R	[g]	853
	PalcA -R	ATACAGCCUTTGAGGCGAGGTGATAGGATTG	CDS-F		
Transcriptional reporter					
mRFP::*Tcyc1Sc*	mRFP-F	ATGGCCTCCUCCGAGGACGTCATCA	promoter-R	[h]	698
	mRFP-R	ATTAAACCTUCTTCGAGCGTCCCAAAACCTTC	B1-F		
	mRFPf-R	AGCGCGAGUCTTCGAGCGTCCCAAAACCTTC	HRS2f-F	[h]	697

PCR templates: [a]pAg1-H3, pRF-HU2 (Frandsen et al. 2008); [b]pPK2 or pPZP-201BK (Covert et al. 2001); [c]pANT-hyg(R) (Fulton et al. 1999) or pCSN43 (Fungal Genetics Stock Center); [d]pAN7-1 (Punt et al 1987); [e]pSM334 (Flaherty et al. 2003) or pTJ-GU2, [f]pBARKS1 (Pall and Brunelli 1993); [g]*A. nidulans genomic DNA* or pRF-HUE, pRF-HU2E (Frandsen et al. 2008),[h]pWJ1350 (Lisby et al. 2003) or plasmids derived from the original *Discosoma* species study (Campbell et al. 2002)

Table 14.2 Design the primers so that they amplify

Purpose	Primer name	Sequence (5' to 3')	Overhang compatible with
Deletion/*in locus* overexpression	HRS1-F[a]	AGGTCGTATU-GSP	B2-R
Deletion/*in locus* overexpression	HRS1-R[a]	AGTATTGCGU-GSP	marker F
Deletion	HRS2-F[b]	ATGACCTAGU-GSP	marker R
Deletion	HRS2-R[b]	ATTAAACCTU-GSP	B1-F
In locus and ectopic expression	CDS-F[c]	AGGCTGTAU-GSP	promoter-R
In locus and ectopic expression	CDS-R[c]	ATTAAACCTU-GSP	B1-F
Transcription reporter	PromoterX-F	ATGACCTAGU-GSP	marker R
Transcription reporter	PromoterX-R	AGGAGGCCAU-GSP	mRFP-F
Expression from fixed locus	CDSf-R	AGCGCGAGU-GSP	HRS2f-F
Expression from fixed locus	HRS2f-F	ACTCGCGCU-GSP	CDSf-R

[a]the natural promoter regions of the target gene, [b]the terminator region of the gene, [c]in case of *in locus* overexpression: the first 1,500 bp of the gene, so that the AU in the forward primer is part of the initial start codon of the target gene, and if the primers are for ectopic overexpression: allow the primer pair to amplify the entire coding sequence plus 500 bp of the terminator

14.5.7.2 Primer Design for the Gene of Interest

For experiments aimed at ectopic expression of the gene with its native regulatory elements: Design a primer pair that amplifies the promoter, coding sequence, and terminator region and append the HRS2-F and CDS-R overhangs to the primers (see Table 14.2 and Fig. 14.1).

For experiments aimed at ectopic expression of a gene controlled by one of the two exogenous promoter elements: Design a primer pair that amplifies the coding sequence of the gene plus 500 bp downstream of the gene (terminator region). The forward primer should amplify the coding sequence from the G in the start codon and addition of the CDS-F 5' overhangs to this primer will create the required start codon of the gene. Add the CDS-R 5' overhangs to the reverse primer (see Table 14.2 and Fig. 14.2).

For experiments aimed at targeted genome modifications: The required size for the homologous recombination sequences (HRSs) for efficient targeted integration into the recipient's genome varies from species to species, in the case of *Fusarium* species we use 1,500 bp. The DNA template for these PCR reactions should be from the recipient organism. For targeted gene replacement/deletion experiments design primers for amplifying two homologous targeting sequences, one on either side of the targeted gene, and append the HRS1-F/R and HRS2-F/R 5' overhangs as specified in Table 14.2, it is important to preserve the relative direction of the two HRSs in the vector to allow for double crossover with the genome (Fig. 14.3).

In case of in locus overexpression experiments (*promoter exchange*) design the first primer pair to amplify the native promoter region (1500 bp) and add the HRS1-F and HRS1-R 5' overhangs to these primers. Design the second primer pair so that the forward primer anneals to the start of the gene, from the G in the start codon (ATG), and the reverse primer anneals approximately 1,500 bp into the genes. There is no need for amplifying the entire coding sequence, as a functional gene will be formed upon integration of the T-DNA via HR. Add the CDS-F 5' primer overhangs to the forward primer and the CDS-R overhang to the reverse primer, as specified in Table 14.2 (Fig. 14.4).

For heterologous expression from a fixed locus in the genome: The system also allows for single step construction of expression cassettes targeted to a specific locus in the recipient fungus genome, as described in (Hansen et al. 2011). The setup can be used to eliminate positional effects on

expression of the gene under analysis, or if a good expression locus has been identified in the host's genome. Design two homologous recombinant sequences, one on either side of the targeted locus, and add the HRS1-F and HRS1-R 5' USER overhangs to the upstream HRS and the HRS2-F and HRS2f-R 5' USER overhangs to the downstream HRS, as specified in Table 14.2 (Fig. 14.5). Design primers for amplifying the gene you want to express including its natural terminator, the forward primer should amplify the coding sequence from the G in the start codon. Add the CDS-F and CDSf-R 5' USER overhangs specified in Table 14.2. During USER cloning include the backbone elements, targeting sequences, one of the two promoter elements, and the gene of interest.

For transcriptional reporter constructs: Constructs of this type allows the analysis of gene expression in situ at a single cell level, based on the detection on the monomeric red fluorescent protein from *Discosoma* species (Campbell et al. 2002). For random integration of the reporter system into the genome, design primers to amplify the promoter region of the gene of interest and add the PromoterX-F and PromoterX-R 5' USER overhangs to the primers, see Table 14.2 (Fig. 14.6). If working in a fungus with a high level of HR, there is a chance that the construct instead of integrating via NHEJ will target the endogenous locus and thereby disrupt the function of the gene.

For targeted integration of the reporter system into the recipient's genome, primer pairs designed for the random integration strategy can be reused by combining the amplicon with different USER-Brick (mRFP-F/mRFPf-R, HRS1, and HRS2), as specified in Table 14.3 (Fig. 14.7).

Table 14.3 USER-Bricks for ectopic integration of a transcription reporter construct

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2e-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
PromoterX-F/R	Promoter to be analyzed	2
mRFP-F + mRFP-R	Fluorescent reporter gene	1

Note the user of the B2e-R primer
[a]Alternatively use the B3-F + B3-R and B4-F + B4e-R backbone USER-Bricks
Remember to add 1 μL USER enzyme mix, 1 μL 10×Taq DNApol buffer and 2 μL MilliQ to the reaction

Table 14.4 Primers for screening and validating the transformants

Primer name	Sequence (5' to 3')	Amplicon	Target
Primers for testing of the selection markers			
HygR-T-F	AGCTGCGCCGATGGTTTCTACAA	588 bp	Marker gene
HygR-T-R	GCGCGTCTGCTGCTCCATACAA		
GenR-T-F	AGCCCATTCGCCGCCAAGTTCT	480 bp	Marker gene
GenR-T-R	GCAGCTGTGCTCGACGTTGTCA		
BAR-T-F	TCAGATCTCGGTGACGGGCA	552 bp	Marker gene
BAR-T-R	ATGAGCCCAGAACGACGCC		
Primers for testing targeted integration			
RF-1 (HygR constructs)	AAATTTTGTGCTCACCGCCTGGAC	[a]	T-DNA
RF-2 (HygR constructs)	TCTCCTTGCATGCACCATTCCTTG	[a]	T-DNA
RF-3 (PgpdA promoter)	TTGCGTCAGTCCAACATTTGTTGCCA	[a]	T-DNA
RF-7 (GenR constructs)	CTTTGCGCCCTCCCACACAT	[a]	T-DNA
RF-6 (GenR constructs)	TCAGACACTCTAGTTGTTGACCCCT	[a]	T-DNA
RF-8 (BarR constructs)	CTGCACTTTTATGCGGTCACACA	[a]	T-DNA
RF-9 (BarR constructs)	CCTAGGCCACACCTCACCTTATTCT	[a]	T-DNA

[a]Depending on the individual construct but larger than the used homologous recombination sequence

14.5.7.3 Primers for Screening and Verification of Fungal Transformants

The standard primers for analysis of the fungal transformants are listed in Table 14.4. In random mutagenesis experiments we typically use two primer pairs, the first for verifying that the marker is indeed present in the transformants (Table 14.4) and a second primer pair targeting the introduced gene. In targeted genome modification experiments we typically rely on three primer pairs for validating that the correct modification has been introduced. The first primer pair targets the used marker gene, the second and third primer pairs targets the borders of the introduced T-DNA (annealing within the selection marker) and these are combined with primers located in the surrounding genome. The primers located in the genome should be designed so that they anneal outside the used HRSs, and amplify part of the genome + HRS + part of the introduced T-DNA by combining them with the relevant RF-1,2,3,7,8, or 9 primers (Table 14.4).

14.6 Methods

In the following section four sub-protocols is presented, each covering a specific step in the AMT process. The first addresses vector construction via the USER-Brick system, the second how to introduce binary vectors into *A. tumefaciens* via electroporation, the third is a generic AMT protocol, and the fourth deals with the identification of correct fungal transformants.

14.6.1 Sub-protocol 1: USER Cloning-Based Construction of Plasmids

The aim of this sub-protocol is to construct AMT compatible vectors via USER cloning. Generally the different USER-Bricks are amplified by PCR using the PfuX7 DNA polymerase and primers containing 2-deoxyuridine. Following amplification and purification (optional), the different USER-Bricks are combined and treated with the USER cloning enzyme mix (NEB) to generate single-stranded compatible overhangs on the fragments. The resulting fragments are then transformed into *E. coli* where the fragments are cova-

Table 14.5 USER-Bricks for ectopic expression of the target gene with its native promoter

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2e-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
HRS2-F + CDS-R	Promoter + CDS + Terminator of target gene	2

Note that the B2e-R primer is used
[a]Alternatively use the B3-F + B3-R and B4-F + B4e-R backbone USER-Bricks
Remember to add 1 μL USER enzyme mix, 1 μL 10×Taq DNApol buffer and 3 μL MilliQ to the reaction

Table 14.6 USER-Bricks for ectopic overexpression of target gene with *PgpdA* or *PalcA* promoters

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2e-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
An_PgpdA-F/R	Constitutive promoter	1
CDS-F + CDS-R	CDS and Terminator of target gene	2

Note that the B2e-R primer is used
[a]Alternatively use the B3-F + B3-R and B4-F + B4e-R backbone USER-Bricks
Remember to add 1 μL USER enzyme mix, 1 μL 10×Taq DNApol buffer and 2 μL MilliQ to the reaction

Table 14.7 USER-Bricks for targeted gene replacement/deletion

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
HRS1-F + HRS1-R	Upstream targeting sequence	2
HRS2-F + HRS2-R	Downstream targeting sequence	2

[a]Alternatively use the B3-F + B3-R and B4-F + B4-R backbone USER-Bricks
Remember to add 1 μL USER enzyme mix, 1 μL 10×Taq DNApol buffer and 1 μL MilliQ to the reaction

Table 14.8 USER-Bricks for *in locus* overexpression of target gene

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
An_PgpdA-F/R	New promoter	1
HRS1-F + HRS1-R	Upstream targeting sequence	2
CDS-F + CDS-R	Downstream targeting sequence (start of the CDS)	2

[a]Alternatively use the B3-F + B3-R and B4-F + B4-R backbone USER-Bricks
Remember to add 1 μL USER enzyme mix, 1 μL 10×Taq DNApol buffer to the reaction

Table 14.9 USER-Bricks for heterologous expression from a fixed locus

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
HRS1-F + HRS1-R	Upstream targeting sequence	2
HRS2f-F + HRS2-R	Downstream targeting sequence	2
An_gpdA-F/R	New promoter	1
CDS-F + CDSf-R	CDS and Terminator of target gene	2

Note the user of the HRS2f-F and CDSf-R primers
[a]Alternatively use the B3-F + B3-R and B4-F + B4-R backbone USER-Bricks
Scale the USER cloning reaction to 15 μL
Remember to add 1.5 μL USER enzyme mix, 1.5 μL 10×Taq DNApol buffer, and 2 μL MilliQ to the reaction

lently linked and replicated. In the following the construction of a vector for targeted gene replacement is used to exemplify the process, meaning that the USER-Bricks and inserts that are mixed in step 3 might vary from the experiment you are doing (see Tables 14.3, 14.5, 14.6, 14.7, 14.8, 14.9, and 14.10 for which USER-Bricks to combine in other types of experiments).

14.6.1.1 USER Cloning Reaction

1. Amplify the required USER-Bricks and gene-specific fragment by PCR, using X7 or PfuTurbo® C_x Hotstart DNA polymerase in a reaction volume of 50 μL per reaction.

Table 14.10 USER-Bricks for ectopic integration of a transcription reporter construct

Primers	Contents	Vol. in USER reaction (μL)
B1-F + B1-R[a]	trfA + ½ KanR	1
B2-F + B2-R[a]	½ KanR + oriV	1
Marker-F/R	Marker cassette	1
HRS1-F + HRS1-R	Upstream targeting sequence	2
HRS2f-F + HRS2-R	Downstream targeting sequence	2
PromoterX-F/R	Promoter to be analyzed	2
mRFP-F + mRFP-R	Fluorescent reporter gene	1

Note the user of the B2e-R primer
[a]Alternatively use the B3-F + B3-R and B4-F + B4e-R backbone USER-Bricks
Remember to add 1 μL USER enzyme mix, 1 μL 10×Taq DNApol buffer, and 3 μL MilliQ to the reaction

Table 14.11 USER cloning reaction

B1-F/R	1 μL
B2-F/R	1 μL
geneY-U1/U2	2 μL
geneY-U3/U4	2 μL
Marker cassette	1 μL
USER enzyme mix	1 μL
10× Taq DNA polymerase buffer	1 μL
MilliQ	*fill up to 10 μL*
Total volume	10 μL

2. Check the success of the PCR reaction by loading 5 μL of the reaction volume on a 1 % agarose gel. Purify the backbone and selection marker USER-Bricks to eliminate the template DNA. For the gene-specific inserts, it is not necessary to purify the PCR amplicon(s), before the USER cloning reaction, if no unspecific bands are detected.
3. USER cloning reaction: Mix the components listed in Table 14.11 in a 0.2 mL PCR tube.
4. Incubate at 37 °C for 25 min followed by 25 °C for 25 min (we use a PCR cycler for this)
5. *Transformation of E. coli*:
   a. Transfer 5 μL of the USER cloning reaction mix to a pre-cooled 1.5 mL Eppendorf tube.
   b. Add 50 μL of chemically competent *E. coli* cells (>1×10^6 cfu) to the Eppendorf tube.

c. Mix gently by tapping on the tube a couple of times and incubate for 30 min on ice.
d. Heat shock the cells by incubating them at 42 °C for 45 s, immediately afterward return the cells to ice and incubate for 2 min.
e. Add 450 μL SOC medium, mix by inverting the tube a couple of times, and incubate for 1 h at 37 °C with shake.
f. Pellet the cells in a tabletop centrifuge (1 min at 1.000 rpm), discard 9/10 of the supernatant, and resuspend the cell pellet by pipetting up and down until all cells have been separated. Plate the approximately 50 μL onto a single LB plate supplemented with 25 μg/mL kanamycin. Incubate the plate overnight at 37 °C.

NB. Electroporation into *E. coli* will not work.

6. The next day: Isolate 5 of the obtained colonies onto a new LB plate supplemented with 25 μg/mL kanamycin and incubate the plate overnight at 37 °C.
7. The next day: Screen 2–5 of the resulting colonies by PCR, using the insert-specific primers used to amplify the two inserts in step 1 (two reactions per colony). It is also possible to perform the screening on the plates from step 6; however, we normally get a very high level of background due to the free amplicon DNA found on the plates from the cloning reaction.
8. Prepare 10 mL liquid LB + kanamycin cultures for the correct colonies and incubate overnight at 37 °C with shake.
9. Purify the plasmid DNA using the Qiagen Miniprep kit or similar
10. Validate correct assembly via restriction enzyme digestion and sequence the insert(s) to eliminate the possibility that the PCR-based amplification has introduced point mutations in the construct.

NB. Identification of correct transformants (step 6–10) can be setup in multiple different ways, in my laboratory some students choose to skip step 6–7 and setup liquid cultures directly from the transformation plates thereby saving a day of incubation. PCR is then performed on the pellet from the liquid culture or on the purified plasmids.

14.6.2 Sub-protocol 2: Transformation of *A. tumefaciens* via Electroporation

Following verification of the binary vector that was constructed via USER cloning, the plasmids should be introduced into an appropriate *A. tumefaciens* strain. The following describes how this can be accomplished via electroporation.

1. Place the electroporation cuvette on ice.
2. Thaw the electro competent *A. tumefaciens* cells on ice.
3. Switch on the electroporation apparatus and change the settings to the following Voltage = 2.50 kV, Capacitance = 25 μF, Resistance = 200 Ω.
4. Pipette 1 μL of your DNA (Miniprep'ed) into the electroporation cuvette.
5. Use 50 μL of competent cells to flush the DNA containing droplet to the bottom of the cuvette.
6. Wipe down the exterior sides of the cuvette with a paper towel (to prevent short circuits).
7. Place the cuvette in the apparatus and shock the cells.
8. Remove the cuvette from the apparatus and add 450 μL SOC medium.
9. Pour the cells into a sterile 1.5 mL Eppendorf tube.
10. Incubate the cells at 28 °C for 90 min with shake.
11. Plate the cells onto two LB + kanamycin plates (1/10 and 9/10 of the volume).
12. Incubate the plates at 28 °C for 2–3 days.

14.6.3 Sub-protocol 3: Generic *A. tumefaciens*-Mediated Transformation Protocol

The following generic protocol can serve as a starting point for optimization of the AMT process. It consists of three phases: pre-culturing, co-culturing, and selection. The optimal incubation time in the different steps varies between different fungal species. The length of the co-culturing step, where the T-DNA is transferred, should not

exceed 3 days, as this will lead to multiple transformation events and hence increase the likelihood that the obtained strains will include multiple T-DNA integrated at different loci. Due to the high number of steps where the plates are handled, there is an increased risk for contamination, why it is advisable to work in a sterile environment (LAF-bench).

14.6.3.1 Pre-culturing of the *A. tumefaciens* Strain

Day 1:

1. Inoculate 10 mL of LB medium (pH >7.7) supplemented with 50 μg/mL kanamycin (and 10 μg/mL rifampicin) in a 50 mL falcon tube with the relevant *A. tumefaciens* strain.
2. Incubate for 2 days at 28 °C with shake at 100 rpm.

Day 3:

1. Prepare 50 mL liquid IMAS-medium (should be fresh)
2. Inoculate 10 mL IMAS + kanamycin (50 μg/mL) in a 50 mL falcon tube with 300 μL of the *A. tumefaciens* LB culture and incubate at 28 °C with shake (80–100 rpm) until OD_{600} reaches 0.5–0.7 (typically the next day).

NB. If *A. tumefaciens* forms filaments or clumps: It is our experience that vortexing the culture vigorously to yield a single cell culture does not negatively affect the transformation frequency.

14.6.3.2 Co-culturing (Transformation Step)

Day 4:

1. Cast eight IMAS plates (five for transformation and three as controls).
2. Place black AGF220 80 mm filters (sterile) onto six of the IMAS plates.
3. Eliminate any air pockets formed between the medium and filter by adding sterile water onto the center of the filters and spreading it with a sterile Drigalski spatula (from the center and out). The amount of water needed depends on the moisture of the plates, but typically we add between 50 and 400 μL. The filters should be moist but not saturated.
4. Allow the *A. tumefaciens* IMAS culture to reach an OD_{600} of 0.3–0.5, vortex culture if necessary.
5. Dilute the fungal spores (or fragmented mycelium) with liquid IMAS-medium to a final spore concentration of 2×10^6 spores/mL.
6. Make the following three control plates:
   1. Fungal spores (2×10^5 spores) (without filter)
   2. *A. tumefaciens* strain alone (without filter)
   3. Sterile filters on IMAS plates
7. Mix the *A. tumefaciens* culture in a 1:1 (v:v) ratio with the fungal spores.
8. Apply 200 μL of the *A. tumefaciens*/fungal mix onto the center of the filters on the IMAS plates (five plates). Spread the solution using a sterile Drigalski spatula. Remember to resuspend the spore/bacterium solution before removing the 200 μL to obtain even distribution of cells.
9. Incubate the plates for 2–3 day at 28 °C in darkness—with the filter side up.

14.6.3.3 Selection

Day 6:

1. Cast five selective DFM plates (or other suiting fungal media) supplemented with 300 μg/mL mefoxin or cefoxitin (kills the *A. tumefaciens* cells) and an appropriate fungicide depending on the used selection marker cassette (For *Fusarium*: 150 μg/mL hygromycin B or 300 μg/mL geneticin or 600 μg/mL DL-phosphinothricin (BASTA)).
2. Under sterile conditions "peel off" the filters and transfer them onto the DFM + mefoxin + fungicide plates using a pair of sterile tweezers. To minimize the formation of air bubbles between the filter and the agar plate, hold the filter vertical and let the bottom part adhere to the surface of the agar plate (near the edge of the plate) and roll the rest of the filter onto the surface in a smooth motion. If large air bubbles are present, the cells on these parts of the filter will not be subjected to selection and hence cause problems later on in the process. Air bubbles can be removed by lifting the filter half way up and then gently rolling it back onto the agar surface.

3. Incubate the five plates at 25 °C for 5 days. The time and temperature will vary between fungal species, but generally use the optimal growth conditions for the fungus at this stage.

Typically day 11:

1. When colonies have spread into the medium, the filters are discarded and visible colonies (star-shaped formation inside the agar) are transferred to new 5.5 cm DFM plates supplemented with appropriate fungicide. Isolate transformants with a wild type phenotype as well as new phenotypes. During the isolation procedure, transfer as little mycelium as possible to reduce the likelihood that you will have a polyclonal culture. The isolation is best done by dipping a sterile toothpick into the edge of a colony (down through the agar) and then repeating the action on the center of the isolation plate, puncturing the agar.

14.6.4 Sub-protocol 4: Identification of Correct Transformants

Following isolation of the fungal transformants, it is often necessary to subculture the transformants several times or make single spore cultures to ensure pure cultures. To be able to identify transformants with the desired genotype, genomic DNA is needed. To ease the screening process, we typically rely on a colony PCR strategy, where mycelium is first heated in a microwave oven and then diluted to eliminate the inhibitory effects caused by cells debris.

14.6.4.1 Colony PCR on Fungi

1. Transfer a small amount of mycelium (ball with a diameter of 1 mm) to 50 μL 10× TE buffer in a 1.5 mL Eppendorf tube with safe-lock.
2. Heat the sample for 5 min at full effect in a microwave oven.
3. Let the sample rest for 5 min at room temperature (25 °C).
4. Centrifuge for 5 min at 10,000 rpm in a table-top centrifuge.
5. Transfer 30 μL of the supernatant to a 0.2 mL PCR tube.
6. Make a 10-times dilution series with MilliQ-water (10, 100, and 1,000 times).
7. Use 1 μL of these dilutions as template for 15 μL PCR reactions with primers targeting the used selection marker gene.
8. For subsequent PCR reactions, use only the dilution that gave the clearest bands.

The strategy for identifying transformants with the desired genotype is highly dependent on the type of genetic modification that was introduced during the AMT process, see primer section for a description. Validation of a transformants genotype and T-DNA copy number is best achieved by performing a Southern blot analysis.

14.7 Conclusion

The presented protocols describe a generic approach for the genetic transformation of filamentous fungi via *A. tumefaciens* mediated transformation. In addition, a robust and efficient protocol for single step construction of binary vectors, via the USER fusion cloning technique, is described. The new USER-Brick system is highly versatile, as a single gene-specific primer amplicon is compatible with two different vector backbones, three fungal selection markers, and two different promoters, allowing for reuse of gene-specific amplicons for several different purposes and vectors with different markers. The described system can easily be expanded and customized to meet the users' needs.

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Agrobacterium tumefaciens-Mediated Transformation of Pucciniomycotina Red Yeasts

15

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15.1 Introduction

The Pucciniomycotina red yeasts include species in the genera *Sporobolomyces*, *Sporidiobolus*, *Rhodotorula*, and *Rhodosporidium* (Aime et al. 2006). The rationales for performing research on these species are numerous. First, these fungi serve as models for understanding gene function in the related rust pathogenic fungi, which are also members of the Pucciniomycotina but cannot be cultured beyond the confines of a host plant. Second, they themselves can serve as biocontrol agents active against plant pathogenic fungi. Third, the red yeasts have a suite of useful attributes, including roles in biodegradation of mycotoxins, biosynthesis of carotenoids, and biofuel production. Lastly, the Pucciniomycotina is a key lineage within the fungal kingdom from which to explore the evolution of this highly successful group of organisms. Surprisingly, however, molecular biology approaches such as DNA transformation have been rarely performed on the Pucciniomycotina. Research in the last few years has now opened the way to generate transgenic strains by at least two methods—biolistic or *Agrobacterium* delivery of DNA—of which the *Agrobacterium* method is described in this chapter.

Multiple unsuccessful attempts were experienced in developing tools for transformation of exogenous DNA into these yeasts. This was due to the lack of Pucciniomycotina-specific gene markers and to the ability of most of them to gain spontaneous resistance to the common drugs used in transformation protocols of Agaricomycotina and Ustilaginomycotina basidiomycete yeasts. Recently, protocols for *A. tumefaciens*-mediated transformation (AMT) of red yeasts became available (e.g. Ianiri et al. 2011, Abbott et al. 2013, Liu et al. 2013). An alternative approach is the use of biolistic delivery of DNA into these species (Ianiri et al. 2011). However, the particle delivery system for biolistics (available solely from Bio-Rad, Hercules, CA) is expensive, requiring a substantial investment. In contrast, the *Agrobacterium* method provides the ability to perform random mutagenesis and targeted gene replacement, and requires the basic resources that are common to a molecular biology or a microbiology laboratory.

Agrobacterium tumefaciens is a soil-borne bacterium that in nature infects plants to cause crown gall disease. A striking feature of *A. tumefaciens* is its natural ability to introduce a short DNA fragment into the plant genome during the infection process. The DNA fragment, called T-DNA (transfer DNA), contains genes

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encoding proteins whose enzymatic products mimic plant hormones, causing undifferentiated growth of the gall. This ability to transfer DNA has been manipulated in the laboratory to allow the bacterium to transfer a variety of DNA molecules into plant cells, and subsequently into other eukaryotic organisms including animal cells, oomycetes, and numerous fungal species. Transformation of fungi with *Agrobacterium* was comprehensively reviewed about a decade ago by Michielse et al. (2005), and since then it has continued to be widely employed to transform fungi (Frandsen 2011). The transformation process is schematically represented in Fig. 15.1.

For the AMT of Pucciniomycotina red yeasts, at least six binary vectors are currently available based on published reports (see Note [1] and Fig. 15.2a). In developing these vectors, and comparing their ability to work in different species, a high degree of specificity was found between the source of gene markers and the species to transform. This specificity seems to be due to intrinsic features of the red yeast itself, the percentage of G+C content in its genome, and/or codon usage (Abbott et al. 2013, Liu et al. 2013). Transformation of red yeasts became successful upon utilization of selectable markers that were native copies of genes and/or native regulatory elements, appropriate G+C content, or recoding the markers or other genes for optimal expression.

[1] Six different binary vectors are available for transformation of Pucciniomycotina red yeasts. Four vectors, pAIS3, pAIS4, pPZPWU3, and pPZPWU5, were generated using the wild type copies of the *URA3* and *URA5* from *Sporobolomyces* sp. IAM 13481 (see Ianiri et al. 2011 for details), and *URA3* and *URA5* of *R. graminis* WP1 (see Abbott et al. 2013 for details) as selection markers. The plasmid pGI3 contains as a selective marker the high G+C content nourseothricin acetyltransferase (*NAT*) gene obtained from *Streptomyces noursei*, placed under the promoter and terminator of the *TUB2* gene of *R. graminis* WP1 (see Abbott et al. 2013 for details). For selection and maintenance of these vectors in bacteria, 50 μg/ml of kanamycin is added to Luria-Bertani medium. The vector pRH2031 features a codon-optimized enhanced green fluorescent protein gene (Rt*GFP*) and hygromycin phosphotransferase gene (*hpt-3*) placed under the control of the promoter and terminator of the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) of *Rhodosporidium toruloides* (see Liu et al. 2013 for details). For selection and maintenance of the vectors in bacteria, 50 μg/ml of rifampicin and 50 μg/ml of spectinomycin are added to the medium.

15.2 Methods

The method for transformation is simple. Fungal and bacterial strains are grown overnight. The following day they are mixed on an induction medium (IM) plate (Fig. 15.1b). Two or three days later the co-culture is transferred from the IM plate onto selective medium. The following section details a procedure for AMT of the *ura5* auxotroph of *Sporobolomyces* sp. This method is the same for use with other selectable markers, by changing the *Agrobacterium* strain, recipient fungal strain/species, and selective medium.

15.2.1 Step 1—Culture *Sporobolomyces* sp. and the Engineered *Agrobacterium* Strains

- Grow an overnight culture of *A. tumefaciens* harbouring the binary vector pAI4 in Luria-Bertani (LB) containing 50 μg/ml kanamycin on an orbital shaker or other shake culture at 22–25 °C.
- Grow an overnight culture in shake flasks at 22–25 °C of the *ura5* auxotroph AIS2 of *Sporobolomyces* sp. in yeast extract peptone dextrose (YPD) medium.

15.2.2 Step 2—*Agrobacterium* and *Sporobolomyces* sp. Cell Preparation

- Concentrate the overnight culture of *Agrobacterium* by centrifuging at 4,000 rpm for 5 min.
- Discard the supernatant.
- Add 8 ml of sterile water to the *A. tumefaciens* cells.
- Spin down the culture at 10,000 rpm for 2 min.

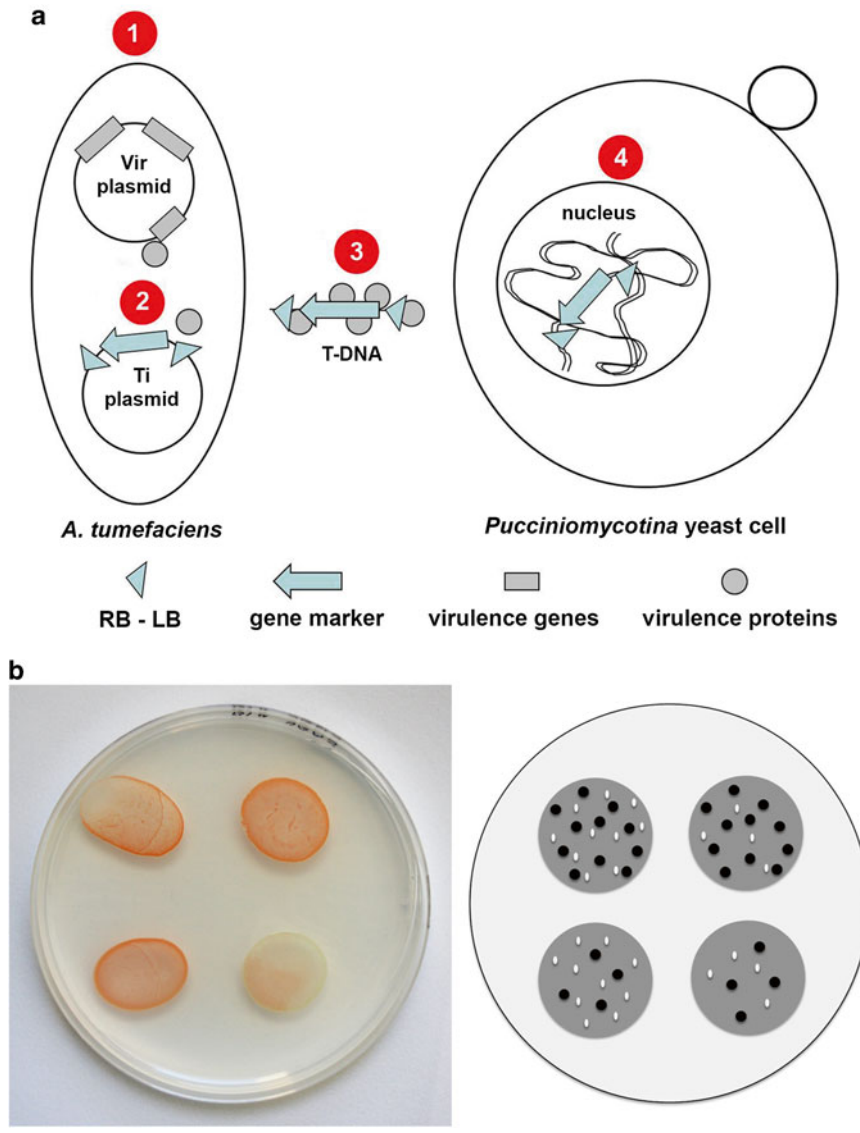

Fig. 15.1 Overview of the *Agrobacterium tumefaciens*-mediated transformation of Pucciniomycotina red yeasts. (**a**) The *Agrobacterium* laboratory strains have been modified to incorporate a binary vector system (1). Vir plasmid contains genes required for virulence (i.e. transfer of DNA into the host). This plasmid will act on the Ti (tumour inducing) plasmid (2), generally called the binary vector and the most commonly manipulated by researchers, where two 25 bp direct repeats, the right and left borders (RB and LB, respectively) enable production of a single strand DNA molecule comprising the gene marker between the two repeats. This DNA is coated with proteins that enable it to be exported from the *Agrobacterium* cell and into the fungal cell (3). The proteins contain nuclear localization sequences, thereby targeting them and the associated T-DNA into the nucleus of the fungus, where integration into the genome occurs (4). (**b**) Photograph of yeast-*Agrobacterium* co-incubation after 3 days (*left*). The initial plating strategy (*right*) involves four mixes of *A. tumefaciens* cells (represented as *white ovals*) and Pucciniomycotina yeast cells (represented as *black circles*) at different concentrations

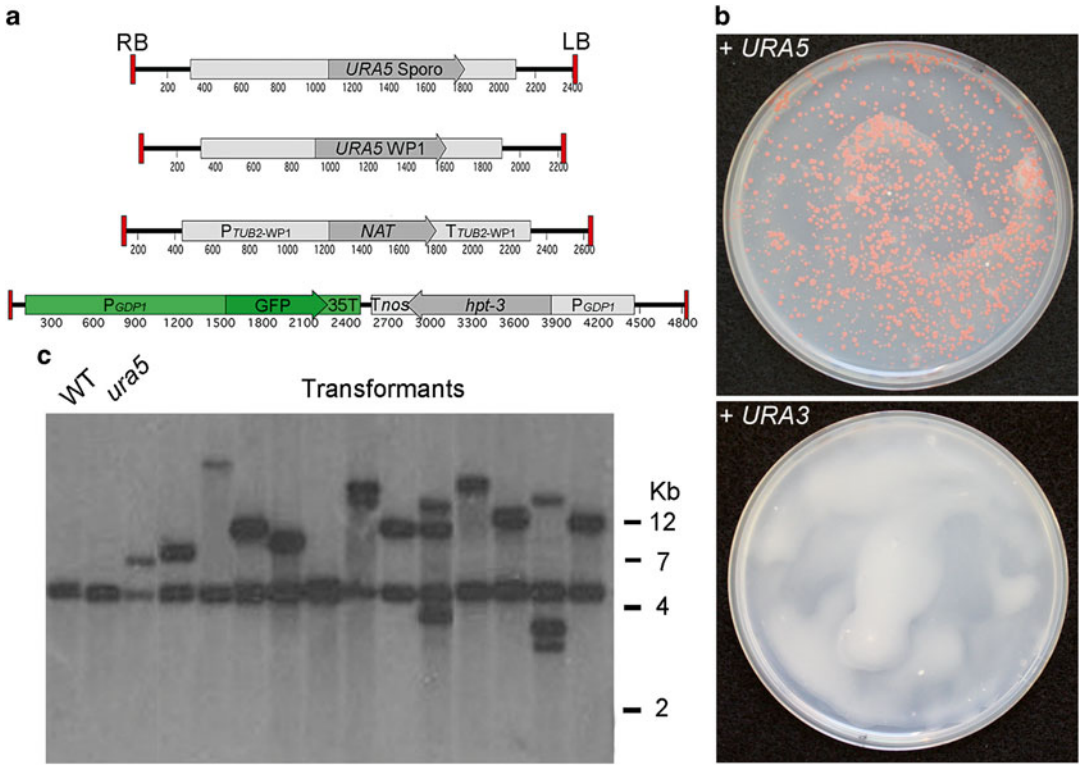

Fig. 15.2 (**a**) Representation of the T-DNAs of the binary vectors pAIS4, pPZPWU5, pGI2, and pRH2031. RB and LB are right and left borders, respectively. The numbers indicate sizes in base pairs. (**b**) Example of selection on yeast nitrogen base medium of transformants of *Sporobolomyces* sp. strain AIS2 (a *ura5* mutant) co-incubated with an *Agrobacterium* strain that delivers the wild type copy of *URA5* (+*URA5*). Two white colonies, presumably bearing T-DNA insertions in the genes for pigment synthesis, are evident. The control plate without any colonies (+*URA3*) was obtained by co-incubating AIS2 with *Agrobacterium* carrying a vector to deliver the *URA3* gene. (**c**) Southern blot analysis of 13 randomly picked transformants derived from *Sporobolomyces* sp. AIS2. The first 2 lanes are the wild type strain (WT) and the *ura5* auxotroph AIS2. The common 4.5 Kb hybridization band represents the original *URA5* locus, while additional bands represent the T-DNA insertions

- Resuspend the cells in 5 ml of liquid IM + acetosyringone [(AS; 3′, 5′-dimethoxy-4′-hydroxyacetophenone), final concentration of 100 μM] (see Note [2]).
- Measure the optical density at 660 nm ($OD_{660\ nm}$) using IM as blank.
- Dilute the culture with liquid IM + acetosyringone to obtain a $OD_{660\ nm}$ of 0.15, in 2–5 ml volume.
- Grow the culture on an orbital shaker at 22–25 °C until the OD_{660} reaches a value of 0.6 (about 4 h).
- Concentrate the overnight culture of *Sporobolomyces* sp. by centrifuging at 4,000 rpm for 5 min.

[2] Make a 2.5x salt solution stock of 2 L H_2O containing: KH_2PO_4 (7.25 g), K_2HPO_4 (10.25 g), NaCl (0.75 g), $MgSO_4 \cdot 7H_2O$ (2.5 g), $CaCl_2 \cdot 2H_2O$ (0.33 g), $FeSO_4 \cdot 7H_2O$ (12.4 mg), $(NH_4)_2SO_4$ (2.5 g). Store at room temperature. To make 1 L of IM, combine 400 ml salt solution, 540 ml H_2O, 5 ml glycerol, and 0.9 g glucose. Agar can be added to make plates (20 g/L). Autoclave, and cool the solution (to 55–58 °C if it contains agar). While cooling, dissolve 7.7 g 2-(*N*-morpholino)ethanesulfonic acid (MES) in 50 ml H_2O. Adjust the pH to 5.3 with 5 M KOH. Add to this solution 19 mg acetosyringone dissolved in 250 μl DMSO. Filter sterilize the MES-AS solution, add to the cooled solution, and pour plates if agar is present. For transformation of uracil auxotrophs, 20 mg of uracil can also be supplemented into the induction medium.

- Add 8 ml of sterile water to the *Sporobolomyces* sp. cells.
- Centrifuge the culture at 10,000 rpm for 2 min.
- Resuspend in 5 ml of IM.
- Calculate the cell concentration using a hemocytometer.
- Adjust *Sporobolomyces* cells to a final concentration of 10^7 cells/ml in IM.

15.2.3 Step 3—Co-incubation of *Agrobacterium* and *Sporobolomyces* sp. Cells

- Prepare four mixes of *Agrobacterium* and *Sporobolomyces* sp. cells as follows (Fig. 15.1b). The rationale for preparing these different ratios is that transformation efficiency is often concentration dependent.
  - 100 µl of induced *Agrobacterium* cells + 100 µl of *Sporobolomyces* sp. cells.
  - 10 µl of induced *Agrobacterium* cells + 100 µl *Sporobolomyces* sp. cells + 90 µl H_2O.
  - 100 µl induced *Agrobacterium* cells + 10 µl *Sporobolomyces* sp. cells + 90 µl H_2O.
  - 10 µl induced *Agrobacterium* cells + 10 µl *Sporobolomyces* sp. cells + 180 µl H_2O.
- Spot mixtures onto IM agar + acetosyringone plates (see Note [3]).
- Allow the plates to dry.
- Incubate the plates, with the lid upwards without parafilm, at 24 °C for 2 or 3 days.

15.2.4 Step 4—Selection of *Sporobolomyces* sp. Transformants

- Scrape the cell mixtures off the IM plates with a disposable cell scraper.
- Transfer the cells into 10 ml of sterilized liquid (e.g. water, phosphate buffered saline, yeast nitrogen base) in a 50 ml Falcon tube.
- Centrifuge 2,000 rpm for 5 min.
- Discard the supernatant, which will remain turbid due to the *Agrobacterium* cells.
- Resuspend the pellet in 1 ml of sterilized water.
- Plate aliquots of 250 µl on selective medium yeast nitrogen base (YNB) + 2 % glucose + 200 µg/ml of cefotaxime (see Note [4]).
- Incubate at 25 °C for 3–5 days until colonies appear (Fig. 15.2b).
- Purify the colonies by re-streaking onto fresh selective plates.

15.2.5 Step 5—Analysis of Transformants

- The subsequent analysis steps depend on the purpose of the transformation experiment. For mutant screens, those with the correct phenotype are identified and genes affected determined, using techniques such as inverse PCR, TAIL PCR, or Splinkerette PCR to amplify the regions flanking the T-DNA insertion. The amplicons are sequenced and compared to the genome database of the strain, if available. Southern blotting can be used to determine the copy number of T-DNA insertions in the transformants (Fig. 15.2c).

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[3] The presence of a physical substrate (e.g. membranes made of cellulose, nitrocellulose, or nylon) on the plates for the co-incubation step was tested, but found to be unnecessary for the success of transformation.

[4] The medium YNB + 2 % glucose is used to select prototrophic strains when the transformation is performed with the *URA* genes as markers. When the gene markers *NAT* and *hpt-3* are used, YPD medium supplemented with 200 µg/ml nourseothricin or 150 µg/ml hygromycin B is used for selection. To prevent *A. tumefaciens* cell growth, 100 or 200 µg/ml of cefotaxime is added to the medium.

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Glass-Bead and *Agrobacterium-Mediated* Genetic Transformation of *Fusarium oxysporum*

16

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16.1 Introduction

Fusarium oxysporum is a ubiquitous soil-borne fungal pathogen which causes vascular wilt in many agriculturally important crops, including banana, cotton, and tomato. The traditional control measures are often less efficient due to its polyphyletic origin and the presence in soil (Di Pietro et al. 2003; O'Donnell et al. 1998). As limited information is available about fungal pathogenesis, diverse molecular approaches are required to identify novel genes for development of fungal resistance in crop plants. An efficient fungal transformation method is required for functional analysis of unexplored pathways/genes by tools like insertional mutagenesis, targeted gene disruption, and RNA interference (Betts et al. 2007; Michielse et al. 2005; Fincham 1989; Kalleda et al. 2013).

Genetic transformation of fungi is conventionally done by homologous recombination-based strategies using protoplasts. Fungal protoplast preparation is a laborious process and is mostly dependent upon batch of enzymes used. Further, low regeneration frequency and reduced DNA uptake by protoplasts significantly limits transformation efficiency (Michielse et al. 2005; Fincham 1989; Meyer et al. 2003). For large-scale mutation studies, a method is required which provides random integration of the gene of interest with high transformation efficiency (Michielse et al. 2005; Michielse et al. 2009).

Agrobacterium tumefaciens is a soil bacterium which can naturally transform the plants by transferring transfer DNA (T-DNA) harboring tumorigenic genes. Ti-plasmid of *Agrobacterium* contains virulence (*vir*) genes and T-DNA; via induction of *vir* genes the T-DNA is transferred from bacterium to plant cell. T-DNA insertion in plant genome is a random process and requires the involvement of host proteins (Zhu et al. 2000; Zupan et al. 2000). In binary vector systems, the T-DNA and *vir* genes are present on separate plasmids for easy genetic manipulations (Hoekema et al. 1983). The trans-kingdom transfer of the T-DNA by *Agrobacterium*-mediated transformation (AMT) was reported in *Saccharomyces cerevisiae* (Bundock et al. 1995). AMT is widely applicable to filamentous fungi and emerges as highly efficient and excellent alternative to conventional transformation methods (Mullins & Kang 2001; de Groot et al. 1998; Michielse et al. 2008).

In *Aspergillus giganteus* transformation efficiencies with AMT are 140 fold higher than the conventional method such as protoplast transformation (Meyer et al. 2003). AMT provides an

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opportunity to use any fungal part like spores, vegetative, and fruiting body mycelia as starting material and also gives higher number of single copy insertions in the fungal genome which is an added advantage for functional studies of genes (Michielse et al. 2005; de Groot et al. 1998; Michielse et al. 2008). Genetic transformation of *F. oxysporum* protoplasts resulted in very low frequencies (Kistler & Benny 1988). However, AMT provides a highly efficient transformation using binary vector, either with fungal-specific (trpC) or CaMV35S-constitutive promoter (Covert et al. 2001; Mullins et al. 2001).

Glass-bead based transformation (GBT) results in higher transformation efficiency as it involves minimal physical damage to the fungal spores and hence more cell survival. Binary vector-based AMT and GBT provide valuable tools for insertional mutagenesis, as T-DNA insertion is a random process and has no similarity with fungal genome. Both AMT and GBT methods have many advantages over other methods of transformation such as simplicity, cost-effectiveness, and no requirement for specialized equipments (Mullins et al. 2001; Feng et al. 2009; Singh & Rajam 2013; Kawai et al. 2010; Zeng et al. 2005).

Here, we describe protocols for the *F. oxysporum* transformation by AMT and GBT methods with some modifications which can also be applied for transformation of other filamentous fungi (Mullins et al. 2001; Singh & Rajam 2013; Minz & Sharon 2010).

16.2 Materials

(*See* Note 1)

1. Sterile distilled water.
2. *Fusarium oxysporum* culture.
3. *Agrobacterium tumefaciens* EHA-105.
4. Glass-beads (0.45–0.52 mm) in diameter.
5. Glass wool.
6. Vortex.
7. Centrifuge.
8. Hemocytometer.
9. Spectrophotometer (OD at 600 nm for bacterial culture).
10. Conc. H_2SO_4.
11. Microfuge tubes.
12. Polypropylene tube (15 mL).
13. Erlenmeyer flask (250 mL).
14. PEG (MW 3500; Sigma, USA).
15. Potato Dextrose Agar (PDA).
16. Potato Dextrose Broth (PDB).
17. 0.1 M Lithium acetate.
18. Nitrocellulose membrane.
19. Liquid nitrogen.
20. Yeast Extract Broth (YEB) medium. For 1,000 mL: yeast extract 1 g, beef extract 5 g, peptone 5 g, sucrose 5 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g. To prepare YEB plates, add bacterial agar to 1.5 %.
21. Antibiotics: Kanamycin (Kan) 100 mg/mL, Hygromycin B (Hyg) 50 mg/mL, and Rifampicin (Rif) 20 mg/mL are prepared in sterile water as stock solutions and stored at −20 °C. The final concentration for antibiotics is 50 mg/L, 100 mg/L, and 20 mg/L respectively. Cefotaxime (Cef) is weighed and used at the concentration of 300 mg/L.
22. Calcium chloride (ice cold) 20 mM.
23. Glycerol 40 %.
24. Plasmid DNA: A binary vector with T-DNA insertion sites should be used. Any commercial vector such as pBin19, pCAMBIA, or equivalent can be used.
25. *Agrobacterium* Minimal Medium (MM). For 1,000 mL: K_2HP0_4 2.05 g, KH_2PO_4 1.45 g, NaCl 0.15 g, $MgSO_4 \cdot 7H_20$ 0.50 g, $CaCI_2 \cdot 6H_2O$ 0.1 g, $FeSO4 \cdot 7H_2O$ 0.0025 g, $(NH_4)_2SO_4$ 0.5 g, glucose 2.0 g (Autoclave) (Hooykaas et al. 1979).
26. MES {2-(*N*-morpholino) ethanesulfonic acid} buffer. Prepare 1 M stock solution, pH 5.5: Titrate to pH 5.5 with 40 % NaOH, filter sterilize, and store at 4 °C under dark condition.
27. 1 M glucose, filter sterilize.
28. 50 % glycerol in water, autoclave.
29. Acetosyringone (AS) 0.1 M in DMSO (store at −20 °C).
30. Induction Medium (IM) Broth. Composed of MM salts (as mentioned above), 40 mM MES buffer, 10 mM Glucose, and 0.5 % Glycerol (Bundock et al. 1995).

31. Induction Medium (IM) Plates. Composed of MM Salts, 40 mM MES buffer, 5 mM Glucose, 0.5 % Glycerol, 200 µM AS, and 1.5 % Agar (Bundock et al. 1995).
32. *F. oxysporum* Minimal Medium (FMM). For 1,000 mL: KH_2PO_4 1 g, Sucrose 30 g, $MgSO_4°7H_2O$ 0.5 g, KCl 0.5 g, $FeSO4 \cdot 7H_2O$ 0.01 g, $NaNO_3$ 2 g, Agar 20 g, Trace elements 0.2 ml (Correll et al. 1987).
33. Trace element stock. For 100 mL: Distilled water 95 mL, Citric acid 5 g, $ZnSO_4 \cdot 7H_2O$ 5 g, $Fe(NH_4)2(SO_4) \cdot 6H_2O$ 1 g, $CuSO_4 \cdot 5H_2O$ 0.25 g, $MnSO_4 \cdot H_2O$ 0.05 g, H_3BO_4 0.05 g, $NaMoO_4 \cdot 2H_2O$ 0.05 g (Correll et al. 1987).

16.3 Methods

16.3.1 Glass-Bead Based Transformation of *F. oxysporum*

16.3.1.1 Fungal Spore Isolation

1. Grow *F. oxysporum* culture on PDA medium at 25 °C for 7 days.
2. Collect the spores from PDA plates by adding 2 mL of distilled water and scrapping it gently with sterile loop.
3. Filter the spore suspension through glass wool.
4. Pellet the spores at 2,000 *g* at 4 °C for 10 min and wash twice with sterile distilled water to remove any mucilage and other remaining spore debris.
5. Resuspend the pellet in sterile water. Count and dilute the spores to the concentration of 1 × 10^6 spores/mL by using hemocytometer.

16.3.1.2 Glass-Bead Based Fungal Transformation

1. Inoculate the spores for germination in PDB for 6 h with shaking at 100 rpm at 28 °C.
2. Sterilize the glass-beads (0.45–0.52 mm) with conc. H_2SO_4, rinsed twice with sterile distilled water and baked at 250 °C for 2–3 h.
3. Add 300 mg of glass-beads to 400 µL of germinated spores along with 1 µg of linearized plasmid DNA (binary vector with desired gene).
4. Subsequently add 400 µl of freshly prepared PEG (60 % W/V) along with 40 µl of 0.1 M lithium acetate (*see* Note 2).
5. Allow the agitation of transformation mixture vigorously at full speed on vortex for 30 s in 15 mL polypropylene tubes.
6. Allow the glass-beads to settle down and then collect the liquid suspension and plate on FMM supplemented with 100 mg/L Hyg in petri plates overlaid with nitrocellulose membrane (*see* Note 3).
7. Keep plates at 28 °C for 6–7 days for appearance of putative transformed fungal colonies.
8. Grow each putative fungal transformants on the separate PDA plates (Hyg) and serially subculture the colonies 4–5 times in PDA (Hyg) plates.
9. Confirm the fungal colonies for integration of gene by PCR and Southern blot analysis.
10. Maintain the single conidial culture of each transformant in 25 % glycerol and store at −80 °C.

16.3.2 *Agrobacterium*-Mediated Transformation of *F. oxysporum*

16.3.2.1 *Agrobacterium* Competent Cell Preparation

1. Inoculate a single colony of *A. tumefaciens* EHA-105 strain in 5 mL of YEB medium for starter culture with 20 mg/L Rif and grow overnight at 28 °C, 250 rpm.
2. Transfer 2 mL of the overnight culture into 100 ml YEB, 20 mg/L Rif.
3. Let it grow to an OD of 0.5–1.0 at 28 °C, 250 rpm. (It takes 8–10 h for the OD to reach to 0.6. All further operations should be done under cold conditions).
4. Chill the culture on ice and pellet the cells at 2,000 *g* for 10 min at 4 °C.
5. Discard the supernatant and resuspend the pellet in 1 mL of 20 mM calcium chloride (ice cold).
6. To store, aliquot 100 µl in sterile, chilled microfuge tubes and add an equal amount of sterile 40 % glycerol and store at −70 °C till use.

16.3.2.2 *Agrobacterium* Transformation by Freeze-Thaw Method

1. Take two vials of stored *A. tumefaciens* EHA-105 strain competent cells and keep them on ice.
2. Add 1 μg of plasmid DNA to one vial and use other as untransformed control.
3. Freeze the cells in liquid nitrogen and thaw by incubating the microfuge tube in a water bath at 37 °C for 5 min till you hear a cracking sound.
4. Keep the vials in ice for 10 min.
5. Add 1 mL of YEB medium to the vials and incubate at 28 °C for 2–4 h with gentle shaking. This period allows bacterial cells to express the antibiotic resistance genes.
6. Centrifuge the vials for 1 min at 2,000 *g* then discard supernatant. Resuspend the cells in 100 μL of YEB medium.
7. Plate the cells on YEB agar plate containing the appropriate antibiotics. Incubate the plate at 28 °C.

16.3.2.3 *Agrobacterium*-Mediated Transformation of *F. oxysporum*

1. Inoculate a freshly isolated single colony of *A. tumefaciens* EHA-105 strain containing the desired binary vector in YEB medium with appropriate antibiotic. Culture overnight at 28 °C with agitation at 250 rpm.
2. Take 1 mL of overnight grown culture and inoculate in 10 mL of YEB medium without antibiotics and agitate at 250 rpm to the OD of 0.4–0.5.
3. Centrifuge the cells at 2,000 *g* for 10 min, remove the medium, and dilute the pellet in IM containing AS (200 μM) to an OD of 0.15 and grow the cells in IM for about 6 h. A negative control without AS can be maintained (*see* Notes 4 and 5).
4. Place sterile nitrocellulose membrane on IM plates. Take 100 μL of the above bacterial culture and mix it with 100 μL (1×10^6 spores/mL) of spore suspension (*see* Subheading 16.3.1.1) in a microfuge tube (*see* Notes 3 and 6).
5. Spread the mixture of *Agrobacterium* cells and fungal spores on the nitrocellulose membrane placed on IM plates containing AS (200 μM) for 2 days (48 h) to cocultivate at 28 °C. Also include a control having only the fungal spores spread on the membrane (*see* Note 7).
6. After the first incubation (48 h), transfer the nitrocellulose membrane to the FMM plates containing 100 mg/L Hyg and 300 mg/L Cef (*see* Notes 8 and 9).
7. Incubate the FMM plates under dark conditions at 25 °C and watch for the appearance of colonies over 5–7 days. No colonies should appear on the control membrane kept on selection plates (*see* Note 10).
8. Count the fungal colonies on the nitrocellulose membrane and calculate the transformation frequency (*see* Note 11).
9. Pick the individual colonies with a sterile loop and transfer each to fresh PDA plates containing antibiotics (Hyg and Cef) (*see* Note 12).
10. Serially transfer the colonies 4–5 times in PDA (Hyg and Cef) to check for stability, growth, and morphology.
11. Isolate genomic DNA from a single conidial culture of each putative transformant and confirm the integration of gene by PCR and Southern blot analysis.
12. Maintain the single conidial culture of each transformant in 25 % glycerol and store at −80 °C.

16.4 Notes

1. Always wear gloves and mask while working with pathogenic fungi and *A. tumefaciens*.
2. Lithium acetate helps DNA to pass through the cell wall so it enhances the efficiency of transformation.
3. Different types of membrane can be used like Hybond, polyvinylidene difluoride, filter paper, nitrocellulose, and cellophane membrane.
4. *Agrobacterium* cell number and pre-growth of *Agrobacterium* cells in the presence of AS

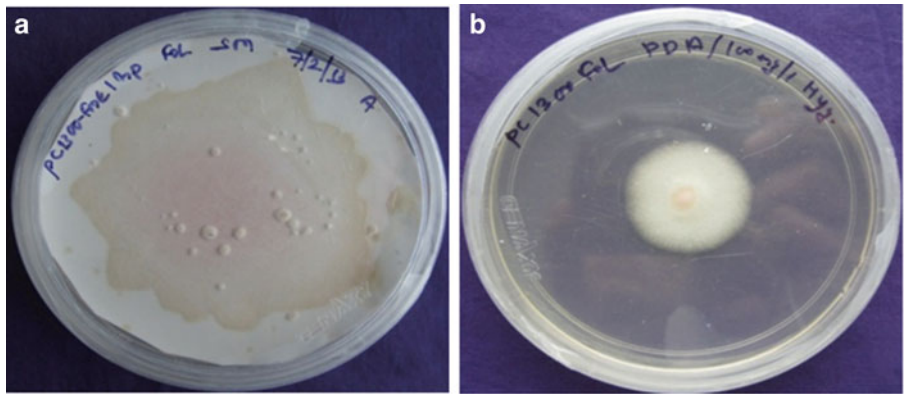

Fig. 16.1 Primary transformants of *F. oxysporum* on the selection medium fortified with 100 mg/L hygromycin B after 5 days (**a**) and 15 days (**b**) of selection

will increase the transformation frequency. A control without AS should always be included. No or few colonies are expected in this control plate.

5. It is better to prepare the IM and the cocultivation medium on the same day of experiment.
6. Freshly isolated and highly viable spores must be used.
7. The fungus should not overgrow on the membrane which might suppress the bacterial growth and reduce the number of transformants. As considerable bacterial growth is required during cocultivation period and is critical for transformation.
8. Effective markers and screening systems are very important in genetic transformation experiments. The common antibiotic markers used in fungi include *hph* (Hygromycin B resistance), *ble* (Bleomycin/Phleomycin resistance), *neo* (Neomycin, Geneticin, and Kanamycin resistance).
9. Hygromycin concentration is critical and stringent selection should be used.
10. Cocultivation time (generally 48 h) in AMT is critical for improving the transformation frequency; if the fungi is fast growing, cocultivation time should be minimum (36 h). Long cocultivation periods can also result in the production of transformants with more than one copy of T-DNA insertion.
11. Selection of individual transformants becomes difficult if they grow in confluence. This problem can be solved by including compounds like Triton X-100 and Na-deoxycholate that reduce growth rate of many fungi and might restrict colony growth in culture.
12. Colonies should be transferred only when clear colonies are seen. False positive colonies may appear after 8 days which may be untransformed.

16.5 Conclusions

GBT and AMT methods described in this chapter are easy to perform, efficient, and cost-effective over the conventional methods of fungal transformation. GBT provides the transformation efficiency of about 15 transformants per μg of plasmid DNA, while AMT results in 150–200 transformed colonies per 1×10^6 spores (Fig. 16.1) as per the protocols developed in the lab using GFP reporter gene construct. These protocols can be used for transformation of other fungi, including recalcitrant fungi for functional analysis of genes.

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Part VI

Transformation Methods: Li-acetate Transformation

17 High Efficiency DNA Transformation of *Saccharomyces cerevisiae* with the LiAc/SS-DNA/PEG Method

R. Daniel Gietz

17.1 Introduction

The term transformation was first coined by Griffith (1928) describing the conversion of the phenotype of *Pneumococcus*, from avirulent to virulent. Transformation of *Saccharomyces cerevisiae* was first accomplished by Hinnen et al. (1978) and Beggs (1978). Ito et al. (1983) first described a method of transforming intact yeast cells utilizing monovalent alkali cations and PEG with a 5-min heat shock at 42°C. Schiestl and Gietz (1989) improved the method by inclusion of single-stranded carrier DNA; however, in 1992 Gietz et al. (1992) showed that the transformation efficiency using the LiAc/ssDNA/PEG method could achieve up to 5×10^6 transformants/μg when certain variables were optimized. Transformation in yeast was reviewed by Gietz and Woods in 2001 (2001) and more recently Kawai et al. (2010).

In the past 12 years much effort has gone into understanding transformation in *S. cerevisiae*. Hayama et al. (2002) published a method of transformation utilizing only PEG as well as a heat shock or pH jump and call this system "natural transformation." The levels of transformation with this method were moderate at best; however, this method still required either PEG or 2,3-Dihexadecanoyl-sn-glycero-1-phosphocholine (PCP) and some form of shock to the cells and seemed to work best if the cells were in early log phase growth. These authors suggest that "natural" transformation occurs during early log phase growth and that this system is distinct from the "chemical" method used to achieve highly efficient transformation. Figure 17.1 shows that bar1Δ yeast cells synchronized in G1 phase using α factor and have a peak in transformation efficiency after release from their cell cycle block at early G1/S phase. Once the cells have transitioned to early S phase the transformation efficiency begins to decline. This suggests that a small window of opportunity during the cell cycle is required to obtain highly efficient transformation. Synchronizing a culture should help increase transformation efficiency and yield.

Kawai et al. (2004) utilized this natural method of transformation to identify yeast mutants that had altered transformation efficiencies when compared to the parent strain. Approximately 5,000 yeast deletion strains were screened for their ability to transform with this "natural" method. The authors identified a number of mutants with reduced levels of transformation and some mutants with increased levels of transformation. These mutants led the authors to suggest that a type of endocytosis is involved in DNA uptake in *S. cerevisiae*. We have also screened for mutants that affect transformation

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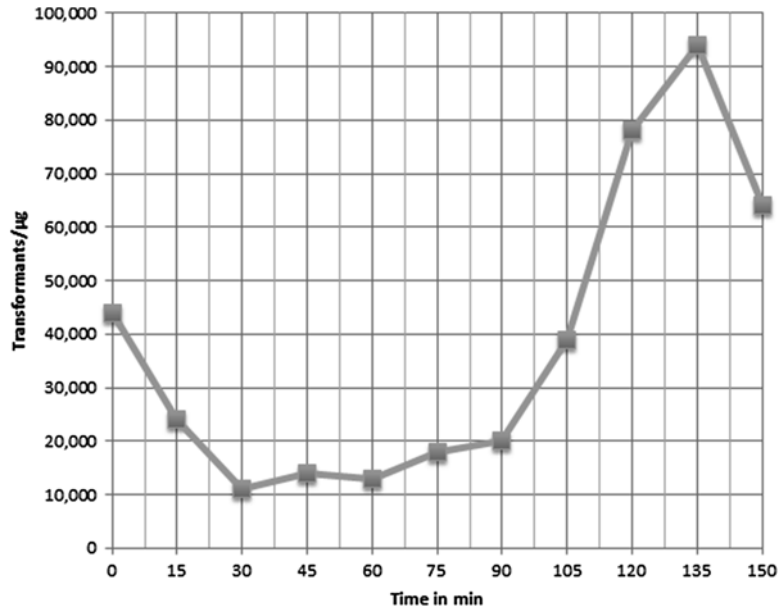

Fig. 17.1 Yeast strain DGY233 (MAT a, ade2-1, lys2-1, ura3-52, leu2-3,112, his3Δ200, trp1-Δ1, bar1-Δ1) was grown overnight in YPAD, diluted 1 in 6 into fresh medium, and treated with alpha factor (Sigma) for 5 h. The arrested cells were washed with sterile H_2O and resuspended in fresh YPAD and incubated at 30°C. Samples were taken every 15 min and the transformation efficiency of each sample (10^8 cells) was determined using plasmid YEplac195. Each sample was also examined under a microscope to identify their progression through the cell cycle. T = 120 is M/G1 and T = 135 is early S phase. (Gietz RD, unpublished results)

utilizing the LiAc/ssDNA/PEG method and our results differ from those presented (Kawai et al. 2004). Table 17.1 shows the mutants, which were identified from a screen of the yeast knock out library purchased from Open Biosystems (Winzeler et al. 1999). In contrast to these authors, we found that *sac6* mutants have 0 % transformation in our system. Six other mutants also were shown to have 0 % transformation when tested in our system (Table 17.1). We also found a number of mutants showing an increased level of transformation efficiency when compared to the parent strain BY4742 (Brachmann et al. 1998). The *snf12* mutant was shown to have the highest transformation rate of all mutants in our system. In addition five other mutants increased the transformation efficiency from 144 to 324 % of the parental strain. It is clear that these mutants do not overlap with those of Kawai et al. (2004), also listed in Table 17.1, which adds strength to the argument that multiple systems of transformation may be at work in *S. cerevisiae*.

Zheng et al. (2005) first demonstrated that the fluorescent dye YOYO-1 could be used to visualize DNA on the surface of the yeast cell. Li + plus PEG was required to induce 99.4 % of cells to bind YOYO-1 labeled DNA. Later it was shown that Li + treatment affected the topography of the cell wall (Chen et al. 2008). In addition it was found that DNA was only bound to the cell wall when PEG was used. We have shown that plasmid DNA labeled with Alexa Fluor 555 could also be visualized on the yeast cell surfaces (see Fig. 17.2a). In addition, labeled DNA, once bound to the cells by the transformation procedure, remains bound to the cells during vegetative cell growth (Fig. 17.2b). Moreover, this fluorescently labeled DNA that is not taken up by the cell can be removed by a micrococcal nuclease treatment (Fig. 17.2c), leaving a subset of cells where this labeled DNA has been internalized making it refractory to nuclease treatment of an intact cell. Kawai et al. (2004) suggested that during DNA internalization PEG acts on the membrane

Table 17.1 Mutants identified screening Open Biosystems yeast deletion collection. Each ORF was replaced with a KanMX antibiotic resistance marker with a unique 20 base pair nucleotide barcode sequence

Gene Name	% of parental strain	Kawai et al. (2004) mutants	Transformation competence
sac6	0	she4Δ	Low
gly1	0	arc18Δ	Low
aat2	0	sin3Δ	Low
pfk1	0	vrp1Δ	Low
caf17	0	las17Δ	Low
ykr041w	0	pan1-9Δ	Low
thr4	0	pan1-20Δ	Low
snf8	144	Spf1Δ	High
mum2	205	Pde2Δ	High
lrp1	217	Pmr1Δ	High
Ybr056w	255		
Ybr053C	324		
snf12	588		

Data from Winzeler E.A. and D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Banghman, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F. Dietrich, S. W. Dow, M. El Bakkoury, F. Foury, S. H. Friend, E. Gentalen, G. Giaever, J. H. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, D. J. Lockhart, A. Lucau-Danila, M. Lussier, N. M'Rabet, P. Menard, M. Mittmann, C. Pai, C. Rebischung, J. L. Revuelta, L. Riles, C. J. Roberts, P. Ross-MacDonald, B. Scherens, M. Snyder, S. Shookhai-Mahadeo, R. K. Storms, S. Véronneau, M. Voet, G. Volckaert, T. R. Ward, R. Wysocki, G. S. Yen, K. Yu, K. Zimmermann, P. Philippsen, M. Johnston, and R. W. Davis. 1999. Functional Characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285: 901-6 and R. D. Gietz, unpublished results

to enhance the transformation efficiency by increasing the permeability of intact cells. However, PEG treatment caused no intracellular response (Kawai et al. 2009). The model proposes that DNA attaches to the cell wall, passes through and subsequently transits the cell membrane by endocytotic membrane invagination. PEG is suggested to be essential for the DNA attachment to the cell wall. DNA enters the cells via endosomal transport but must escape degradative targeting to the vacuoles and enter the nucleus for the cell to become truly transformed. In addition, visualizing vector DNA with a fluorochrome label to transformation of yeast cells containing a GFP fusion gene showed that it localizes to a specific yeast cell compartment (Huh et al. 2003, Fig. 17.3). This clearly suggests that some labeled internalized DNA seems to co-localize to the endosome compartment labeled with GFP.

Pham et al. (2011a) showed that the 42°C heat shock was important only to intact cell transformation as spheroplasts did not respond to it. These authors also used negatively charged Nanogold particles in an attempt to study the uptake of DNA into the cell. They observed many Nanogold particles associated with yeast cell wall and membrane as well as some associated with invaginating membranes.

Similar Nanogold particles were used to understand the synergistic effect of LiAc, and ssDNA on transformation efficiency (Pham et al. 2011b). After treatment of cells with LiAc and ssDNA or RbAc and ssDNA, the 42°C heat shock caused the Nanogold particles to be associated with the cell wall, in many cases being trapped inside the cell wall. In addition, these authors suggest that only DNA bound to the cell wall is available for transformation and both ssDNA and LiAc act to modify the yeast cell wall. While much of this model of transformation is reasonable, the role of ssDNA in the transformation reaction is likely nothing more than a quenching agent for the vast number of DNA binding sites that are found on the yeast cell wall after treatment with LiAc and PEG. PEG acts, probably by molecular exclusion, to deposit all large molecular weight DNA and ssDNA and/or RNA onto the surface of the yeast cell wall (Gietz et al. 1995, Gietz and Woods 2001). There are two types of DNA binding sites found on the yeast cell wall: (a) productive, able to give rise to transformants and (b) nonproductive, unable to give rise to transformants. The large quantity of ssDNA floods nonproductive binding sites, allowing plasmid DNA to be more efficiently bound on the productive binding sites and not trapped on nonproductive binding sites, unavailable for transformation. The treatment of cells with LiAc/ssDNA/PEG with a 42°C heat shock causes the structure of cell wall to be altered and DNA bound to the productive DNA binding sites is taken into the cell via endocytosis. Most importantly, the DNA must escape the traditional endosome pathway to allow it to enter the nucleus.

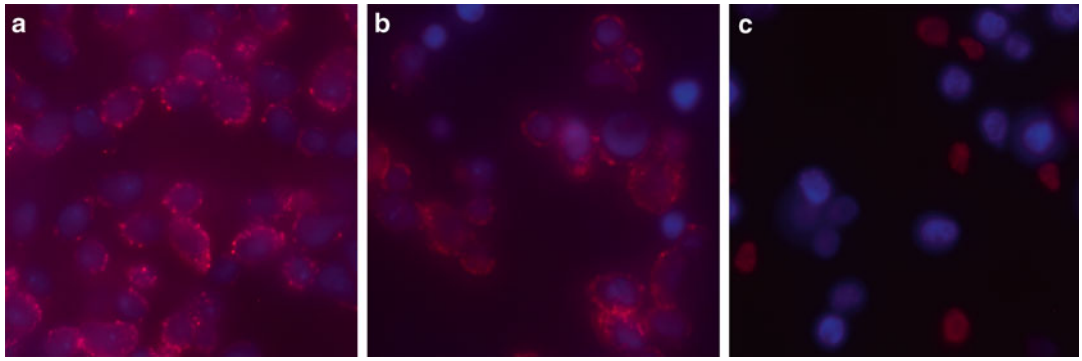

Fig. 17.2 Yeast transformed with plasmid YEPlac195 labeled with Alexa Fluor 555. Yeast cells were imaged using a Zeiss AxioplanII microscope equipped with a Hamamatus CCD camera. Yeast cells were transformed with 100 ng of plasmid DNA labeled with Alexa Fluor 555 using the high efficiency protocol, fixed with formalin, and then stained with DAPI and visualized using a 63× or 40× lens, (**a**) yeast cells after standard transformation (630× magnification). (**b**) Yeast cells transformed with labeled plasmid. Cells were returned to YPAD medium and incubated at 30°C for 48 h with shaking and then a sample of cells was imaged at 630× magnification. (**c**) Yeast cells transformed with labeled plasmid DNA. Prior to fixation the cells were resuspended in micrococcal nuclease buffer and digested with 5 units of micrococcal nuclease for 15 min at 30°c. The cells were then washed in water, fixed and imaged (400× magnification)

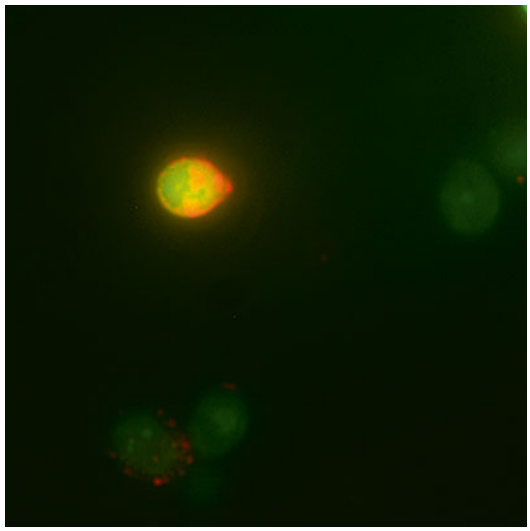

Fig. 17.3 Transformation of endosome GFP strain YLR025W with Alexa Fluor 555 labeled YEPlac195. Yeast cell transformed with labeled plasmid DNA. Prior to fixation the cells were resuspended in micrococcal nuclease buffer and digested with 5 units of micrococcal nuclease for 15 min at 30°c. The cells were then washed in water, fixed and imaged (630× magnification)

Here, four methods for the transformation of *S. cerevisiae* are provided. The Quick and Easy transformation method (Gietz and Schiestl 2007a) can be used to give a few hundred transformants when only a few transformants are needed. The High Efficiency Transformation method (Gietz and Schiestl 2007b) can be used to produce millions of transformants. The microtiter plate transformation method can be used for efficient transformation in a 96 well format (Gietz and Schiestl 2007c). Finally, I have included a method for the production of frozen competent yeast cells (Gietz and Schiestl 2007d) that can be prepared in advance and used with high efficiency at a moment's notice.

17.2 Materials

17.2.1 YPAD Medium

YPAD (Yeast Extract-Peptone-Adenine-Dextrose) is used for routine growth of yeast strains prior to transformation as many strains contain the *ade2* mutation and grow more vigorously when given adenine. Double strength YPAD broth (2xYPAD) is used to grow cultures to log phase before transformation. Recipes for YPAD and 2xYPAD can be found in Gietz and Woods (2006). These media should be supplemented with adenine hemisulphate at a concentration of 0.1 mg/mL. G418 resistance can be used to select for transformation (Shoemaker et al. 1996).

17.2.2 SC Selection Medium

SC (Synthetic Complete) selection medium is used for selection of nutritional genetic markers (Gietz and Woods 2006).

17.2.3 Lithium Acetate (1.0 M)

Dissolve 51.0 g of lithium acetate dihydrate (Sigma Chemical Co. Ltd., St Louis, MO. Catalogue # L-6883) in distilled/deionized H_2O and make up to 500 mL, sterilize by autoclave and store at room temperature.

17.2.4 PEG MW 3350 (50 % w/v)

Add 200 g of PEG 3350 (Sigma Chemical Co. Ltd., Catalogue # P-3640) to 120 mL of ddH_2O in a 1 L beaker. Dissolve on a stirring plate. Make the volume up to exactly 400 mL in a graduated cylinder and mix by inversion. Transfer the solution to a storage bottle and autoclave to sterility and stored at room temperature. Ensure your bottle is capped well to prevent evaporation, which will severely affect the yield of transformants.

17.2.5 Single-Stranded Carrier DNA (2.0 mg/mL)

Dissolve 200 mg of salmon sperm DNA (Sigma Chemical Co. Ltd., Catalogue # D-1626) in 100 mL of TE (10 mM Tris-HCl, 1 mM Na_2 EDTA, pH 8.0). You can use a magnetic stir bar over night at 4°C to ensure good dissolution. Aliquots should be stored at –20°C. Carrier DNA should be denatured in a boiling water bath for 5 min and chilled immediately in an ice/water bath before use. Single-stranded carrier DNA can be boiled 3 or 4 times without loss of activity.

17.2.6 General Equipment

General microbiological supplies are required are listed here. A microtiter plate centrifuge is required for the microtiter plate transformation method. A microtiter plate replicator (Fisher Scientific, Cat# 05-450-9) and a multi-channel micropipettor (Eppendorf™) are also required for the microtiter plate transformation protocols. In addition for the microtiter plate method the plates must be shaken (not stirred) using a rotary shaker. A microtiter plate holder can be fashioned from 1/4 in. plywood or plexiglass by cutting out microtiter plate size rectangles. The plates (plus lids) should fit the slots with minimal play.

17.3 Methods

17.3.1 Quick and Easy Transformation Method

1. Inoculate choice of yeast strain onto an YPAD agar plate or in 2 mL of YPAD liquid medium and incubate overnight at 30°C. [1]
2. The following day prepare single-stranded carrier DNA in a boiling water bath for 5 min and chill in ice/water. [2]
3. Scrape a 50 µL blob of yeast cells from the YPAD plate using a sterile loop or toothpick and suspend the cells in 1 mL of sterile water in a microcentrifuge tube. The suspension should contain about 5×10^8 cells. Alternatively, spin down the 2 mL culture and resuspend in 1 mL of sterile water as above.
4. Pellet the cells at top speed in a microcentrifuge for 30 s and discard the supernatant.
5. Add the following components to the pellet in the following order; 1) 240 µL PEG 3500 (50 % w/v), 2) 36 µL lithium acetate 1.0 M, 3) 50 µL SS carrier DNA (2.0 mg/mL), 4) 34 µL plasmid DNA plus dd H_2O.[3]

[1] This method can be used on yeast cells in different stages of growth and storage; however, yield will be reduced when compared to freshly grown cells.

[2] Carrier DNA is stored in 1.5 ml microcentrifuge tubes. Before denaturation in a boiling water bath pierce the top with an 18 gauge or smaller needle to keep the top from popping.

[3] The addition of DMSO to the Transformation Mix can increase the yield of transformants with some strains. The strain Y190 shows a tenfold increase when 5 % (v/v) DMSO was added to the Transformation Mix.

6. Resuspend the cell pellet by briskly vortexing.
7. Incubate the tube in a water bath at 42°C for at least 20 min. [4]
8. Centrifuge the transformation tube at top speed for 30 s and discard the supernatant.
9. Resuspend the cell pellet in 1 mL of sterile water. Use the pipette tip to disrupt the cell pellet, which will aid in resuspension.
10. Plate 200 µL samples of the cell suspension onto five plates with appropriate selection medium. Transformants can usually be isolated after incubation of 3 or 4 days at 30°C.

17.3.2 High Efficiency Transformation Method

1. High efficiency transformation requires freshly grown yeast cells for best results. Inoculate choice of yeast strain into 5 mL of 2x YPAD or 20 mL of the appropriate selection medium and incubate overnight (16 h) at 30°C on a rotary shaker at 200 rpm. To ensure minimal growth lag pre-warm a culture flask with the medium for the next step.
2. The following day, determine the titer of the yeast culture using one of methods below.
   a) Dilute 10 µL of culture into a final volume of 1 mL sterile water (1/100 dilution), mix thoroughly and measure the OD at 600 nm (a suspension containing 1 × 10^6 cells/mL will give an OD_{600} of about 0.1). [5]
   b) Dilute 100 µL of culture into a final volume of 1 mL of sterile water (1/10 dilution) in a microcentrifuge tube and mix thoroughly. Deliver 10 µL onto the counting grid of an improved Neubauer hemocytometer, wait several minutes for the cells to settle, and count the number of cells in the 25 large grid squares. [6]
3. Add 2.5 × 10^8 cells to 50 mL of the pre-warmed 2x YPAD in the pre-warmed culture flask. The titer should be 5 × 10^6 cells/mL. Alternatively, the titer can be checked after dilution.
4. Incubate the culture in the shaking incubator at 30°C and 200 rpm until the cell titer is at least 2 × 10^7 cells/mL. This can take about 4 h and at times longer with some strains.
5. Prepare carrier DNA by denaturation in a boiling water bath for 5 min and chill immediately in an ice/water bath. [7]
6. Harvest the cultured cells once they have reached the correct titer by centrifugation at 3,000 *g* for 5 min, wash twice with 25 mL of sterile water and resuspend the cells in 1 mL of sterile H_2O.
7. Pellet the cells in a fresh 1.5 mL microcentrifuge tube by centrifugation at maximum speed for 30 s and discard the supernatant.
8. Resuspend the cell pellet in 500 µL of sterile ddH_2O and transfer 50 µL samples containing 10^8 cells into fresh 1.5 mL microcentrifuge tubes for each transformation. Pellet cells at top speed for 30 s in a microcentrifuge and remove the supernatant.
9. Add the Transformation Mix to each tube containing cell pellet and resuspend the cells by vigorous vortexing. Transformation mix is made up prior to this step and stored on ice and the following components added in the

[4] This heat shock will result in several thousand transformants per tube. Extending the duration of the heat shock up to 60 min can increase the yield of transformants in some strains significantly. Consider testing each strain to identify the optimal heat shock time.

[5] When calculating the titer, do not forget your dilution factor (1/100).

[6] The counting grid is made up of 25 large squares bounded by triple lines; each large square is subdivided into 16 small squares bounded by single lines. The total volume of the counting area is 0.1 µL therefore multiply the cell number after counting all 25 squares by 10,000 and the dilution factor (10x) to get cells/mL.

[7] Carrier DNA previously denatured can be stored at –20°C and used 2–3 times without having to denature it again.

following order and then mixed on a vortex mixer: 1) PEG 3500 (50 % w/v) 240 µL, 2) lithium acetate 1.0 M 36 µL, 3) SS carrier DNA (2.0 mg/mL) 50 µL, 4) plasmid DNA and sterile ddH_2O 34 µL. Make additional aliquots calculating the number of transformations planned.

10. Place the tubes in a 42°C water bath for 40 min.[8]
11. Pellet the cells in a microcentrifuge at top speed for 30 s and remove the Transformation Mix. Use a pipettor to remove as much of the Transformation mix as possible.
12. Resuspend the cell pellet in 1 mL of sterile ddH_2O. This can be difficult therefore add a small amount of sterile ddH_2O and stir the pellet with a micropipette tip to aid in suspension of the cells followed by vigorous vortexing.
13. Plate the resuspended cells onto the appropriate selection medium. A good transforming strain will give up to 2×10^6 transformants/µg plasmid DNA/10^8 yeast cells. Plate 2, 20, and 200 µL onto the appropriate selection medium. [9]
14. Incubate the plates at 30°C for 3–4 days to recover transformants.

This method can be used to generate large numbers of transformants required to screen complex clone libraries such as a two-hybrid or similar screens. It is advisable to test the effects of increasing plasmid DNA on transformation efficiency before embarking on a large screen. This information will allow the determination of the appropriate scale up factor (30×, 60×, or 120×) and the appropriate plasmid amount to obtain the number of transformants required to cover the DNA library complexity with high probability. Specific considerations for these screens are found in Gietz (2006).[10, 11, 12]

17.3.3 Microtiter Plate Transformation Method

A method for the transformation of yeast cells in 96 well microtiter plate format is presented here. Sterile 96 well microtiter plates with round bottoms and lids are used for this method. The Microtiter Plate Protocols can be adapted for a number of purposes. A) Many different yeast strains can be grown on a master plate, sampled with a replicator into the wells of a microtiter plate and tested for transformation efficiency with a single plasmid. B) A single strain can be transformed with many different plasmids (e.g., a plasmid library in a 96 well format). C) Many yeast strains can be grown on a master plate, transferred to wells containing 150 µL of 2xYPAD, re-grown in sealed plates on a shaker at 200 rpm, and then transformed *in situ* with a single plasmid. A 96-prong replicator and 150 mm petri dishes of medium are used for this method.

[8] This heat shock will result in several thousand transformants per tube. Extending the duration of the heat shock up to 120 min can increase the yield of transformants in some strains significantly. Consider testing each strain to identify the optimal heat shock time.

[9] Plating volumes of less than 100 µL should be plated into a 100 µL puddle of sterile ddH_2O.

[10] Two-hybrid screens require the transformation of both "bait" and "prey" plasmids into a specific yeast strain. This can be done sequentially or together; however, the best transformation yields are often obtained with a sequential transformation approach.

[11] A two-hybrid screen can be accomplished by transforming the "bait" plasmid into the yeast strain. This strain can then be used to test various amounts (0.1, 0.5, 1.0, 2.0, 5.0, and 10 µg) of prey plasmid library using the High Efficiency Transformation Protocol. This will allow the estimation of the amount of library plasmid and the scale up needed to cover or approach the library complexity with high probability.

[12] The "bait" plasmid and the "prey" plasmid library can be co-transformed into the yeast strain in a single operation. The transformation efficiencies of co-transformation are up to 40 % of the number of transformants from a single high efficiency transformation. Co-transformation may be necessary if the "bait" plasmid affects the growth or viability of your yeast strain.

The Transformation Mix for these protocols is prepared without the PEG reagent making the cell pellet easier to resuspend. The PEG is added after the cell pellets have been resuspended. This method can use an agar plate method or a liquid method for growth of the cells to be transformed depending on your specifications.

Table 17.2 Microtiter plate transformation mix volumes

Component	Per well	Per plate
Lithium acetate 1.0 M	15.0 µL	1.5 mL
SS carrier DNA (2 mg/mL)	20.0 µL	2.0 mL
Plasmid DNA (20 ng) + ddH_2O	15.0 µL	1.5 mL
Total volume	50.0 µL	5.0 mL

17.3.3.1 Agar Plate Method

1. The 96 well replicator prongs are sterilized by dipping in a petri plate containing 70 % ethanol and then passing it through a Bunsen flame and cooling. [13]
2. Carefully set the cooled prongs of the sterile replicator onto the surface of a 150 mm YPAD plate. This will print the position of each well on the agar plate.
3. Patch the yeast strain(s) onto the positions as necessary. Be sure to mark the orientation of the master plate and incubate overnight at 30°C.
4. The following day pipette 150 µL samples of sterile water into each well of the microtiter plate.
5. Denature carrier DNA (2 mg/mL) for 5 min in a boiling water bath and chill in ice/water.
6. Cool the sterilized replicator by dipping prongs into microtiter plate containing sterile water.
7. Place the prongs of the cooled replicator onto the plate, making sure that each prong contacts a patch of yeast inoculum. Gently move the replicator in small circles to transfer cells to the prongs taking care not to cut into the agar surface. Remove the replicator and inspect the prongs for yeast cell coverage.
8. Place the replicator into the microtiter plate containing the sterile water and agitate in a circular motion to wash the cells off the replicator prongs. This will give approximately 1×10^7 cells per well. Repeating the transfer process will increase the number of cells, if necessary. Mark the orientation of the microtiter plate.
9. Pellet the cells by centrifugation 10 min at 1,300 *g* using a microtiter plate rotor.
10. Remove the supernatant from the wells. This may be accomplished by aspiration or dumping in a sink followed by a sharp flick to remove the last remaining drop. This technique should be practiced prior to using it on a screen.
11. Mix the Microtiter plate Transformation Mix as indicated in Table 17.2. The volumes listed are for a single transformation (each well). Make sufficient for 100 transformations if you intend to use all 96 wells. The plasmid amount can be increased but the volume must stay the same.
12. Deliver 50 µL of the Microtiter plate Transformation Mix into each well. Secure the microtiter plate to a rotary shaker at 400 rpm for 2 min to resuspend the cell pellets.
13. Pipette 100 µL PEG 3350 (50 % w/v) into each well and place back onto rotary shaker for 5 min at 400 rpm to mix the PEG and cell suspensions.
14. Place each microtiter plate into plastic bag or seal with Parafilm™ and incubate at 42°C for 1–4 h. [14]
15. Centrifuge each microtiter plate for 10 min at 1,300 *g*, remove the supernatant by aspiration, and resuspend the cells by adding 50 µL of sterile water to each well followed by placement on a rotary shaker at 400 rpm for 2–5 min. Microtiter plate wells may be

[13] Care should be taken when sterilizing the 96 well replicator with ethanol and open flame. Ensure the replicator wet with ethanol is held carefully away from any items before passing through the flame. Hold with prongs hanging down for 60 s to cool.

[14] After incubation at 42°C for 60 min we have obtained an efficiency of 2×10^5 and a yield of 570 transformants per well; extending the incubation to 4 h resulted in an efficiency of 3.9×10^6 and 6200 transformants per well.

sampled individually by sampling a 5 µL aliquot from a well into 100 µL puddles of sterile water on plates of selection medium. A sample can be taken using the replicator to print samples (ca; 5–10 µL) onto selection plates. Multiple samples can be delivered onto large plates using the replicator if care is used to print samples to the exact positions. Incubate the plates at 30°C for 2–4 days and recover the transformants.

17.3.3.2 Liquid Culture Protocol

The yeast cells of the re-grown culture are harvested, washed, and resuspended in water and the cell titer determined as described earlier (17.3.2.2.).

1. Adjust the titer of the cell suspension to 5×10^8 cells/mL and dispense 100 µL of the suspension into the wells of the microtiter plate.
2. Continue from step 9 of the agar plate protocol but increase the amount of plasmid to 100 ng/transformation.
3. Seal and incubate the plates at 42°C for 60 min.
4. Sample the wells using a pipette or microtiter replicator onto selection medium.
5. Incubate the plates at 30°C for 2–4 days and recover and/or count the transformants.

17.3.4 Transformation-competent Frozen Yeast Cells

This method can be used to produce frozen competent yeast cells when a single yeast strain is used repeatedly. Yeast cultures are re-grown for at least two divisions and used to produce transformation-competent cells that are frozen and used at a moment's notice.

17.3.4.1 Preparation

1. The yeast strain is grown overnight and then re-grown in 2x YPAD to a titer of 2×10^7 cells/mL as described in 17.3.2. One hundred samples of 1×10^8 frozen competent cells will require 500 mL of re-grown culture (1×10^{10} cells).
2. Harvest the cells by centrifugation at 3,000 *g* for 5 min, wash the cells in 0.5 volumes of sterile water, and resuspend in 5 mL of sterile water. Transfer to a suitable sterile centrifuge tube and pellet the cells at 3,000 *g* for 5 min.
3. Resuspend the cell pellet in 5 mL of frozen competent cell (FCC) solution (5 % v/v glycerol, 10 % v/v DMSO). Use high quality DMSO for best results.
4. Dispense 50 µL samples into an appropriate number of 1.5 mL microfuge tubes.
5. Place the microfuge tubes into a 100 tube Styrofoam rack with lid (Sarstedt #95.064.249) or similar type of rack. It is best to place this container upright in a larger box (Styrofoam or cardboard) with additional insulation such as foam chips or newspaper to reduce the air space around the samples. This will result in the samples freezing slowly, which is essential for high survival rates.
6. Place the container at –80 °C overnight. The tubes can then be removed from the tube rack container and stored at –80°C in bulk. These cells can be stored for up to 1 year with little loss of transformation efficiency.

17.3.4.2 Transformation of Frozen Competent Yeast Cells

These cells are transformed using a modified High Efficiency Transformation method 3.2 with the differences listed below.

1. Thaw cells in a 42°C water bath for 15 s.
2. Pellet the cells at 10,000 *g* in a microcentrifuge for 2 min and remove the supernatant.
3. Add 360 µL of FCC transformation mix (260 µL 50 % PEG, 36 µL 1.0 M LiOAc, 50 µL denatured carrier DNA, and 14 µL of DNA and water) and vortex vigorously to resuspend the cell pellet. Note the difference in PEG volume.
4. Incubate in a 42°C water bath for 20–60 min depending on the strain. Centrifuge as above to remove the supernatant and resuspend the cell pellet in 1 mL of sterile water.
5. Plate appropriate dilutions onto selection medium.

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18 Transformation of Intact Cells of *Saccharomyces cerevisiae*: Lithium Methods and Possible Underlying Mechanism

Shigeyuki Kawai and Kousaku Murata

18.1 Introduction

Before the initial publication of the lithium method in 1983 (Ito et al. 1983), it is hard to imagine that how intact cells of *Saccharomyces cerevisiae* could be transformed, which are surrounded by rigid and thick cell walls. However, the developed lithium method allowed for the first time the successful transformation of intact *S. cerevisiae* cells using plasmid DNA (Ito et al. 1983). This monumental method represented a significant advance in genetic and biological studies of yeast, and has also contributed to the rapid analysis of the genes of higher animals and plants. Subsequently, the method has been modified in several ways. Here, we describe the principles of the original and modified lithium methods, a possible mechanism underlying transformation of intact cells, and finally the modified lithium method that we currently use.

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18.1.1 Original Lithium Method

In the original lithium method (Ito et al. 1983), a most important factor that increased the transformation efficiency (number of transformants per μg plasmid DNA) of intact *S. cerevisiae* cells was the presence of monovalent cations such as Na^+, K^+, Rb^+, Cs^+, and especially Li^+. It is surprising that divalent cations such as Ca^{2+}, which is effective in *Escherichia coli* transformation, are not effective for transformation of intact *S. cerevisiae* cells. Lithium was tested because it is effective in separation of inorganic polyphosphate, a negatively charged macromolecule similar to DNA, from anion-exchange columns (Kawai et al. 2010). In addition to the use of lithium, the original lithium method has several important features: (i) incubation of intact cells with both polyethylene glycol (PEG) and plasmid DNA is essential for transformation; (ii) short-term incubation of intact cells with PEG and plasmid DNA at 42 °C (heat shock) increases the transformation efficiency; and (iii) transformation of cells harvested at mid-log phase is most efficient (Ito et al. 1983). PEG was tested in the original lithium method because this reagent is used in the spheroplast method (Hinnen et al. 1978). The original lithium method yielded about 450 transformants/μg of plasmid DNA (Ito et al. 1983).

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18.1.2 Modified Lithium Methods

Based on the principles established in the original lithium method (Ito et al. 1983), Gietz and co-workers succeeded in improving the efficiency to 5×10^6–1×10^7/μg of plasmid DNA from 10^8 cells by immediately mixing washed intact cells with PEG, lithium acetate (LiAc), plasmid DNA, and single-stranded carrier DNA (ssDNA), and then incubating them at 42 °C for 40–60 min (Gietz et al. 1992, Gietz et al. 1995, Gietz and Woods 2002, Schiestl and Gietz 1989). Their protocol has been referred to as the LiAc/ssDNA/PEG method (Gietz and Woods 2002).

In contrast to the approach of Gietz et al., who added components to the original lithium method, we removed LiAc from the original method, and found that intact cells could be transformed by incubating cells with only PEG and plasmid DNA at 30 °C and then at 42 °C (heat shock) (Hayama et al. 2002). This method was tentatively called the "natural transformation method" (Hayama et al. 2002).

18.1.3 Possible Mechanism Underlying Transformation of Intact Cells

We evaluated the effect of ssDNA and LiAc on transformation efficiency in the LiAc/ssDNA/PEG method, and observed that LiAc and ssDNA synergistically improved the transformation efficiency of intact *S. cerevisiae* cells (Table 18.1) (Pham et al. 2011b). Using transmission electron microscopy (TEM), we observed *S. cerevisiae* cells incubated with PEG and negatively charged Nanogold (in this context, a mimic of DNA) in the presence of no additional reagents (Fig. 18.1a), ssDNA (Fig. 18.1b), LiAc (Fig. 18.1c), and both LiAc and ssDNA (Fig. 18.1d). Together, LiAc and ssDNA made the cell wall form extremely protruded, loose, and porous structures (Fig. 18.1d); LiAc alone caused the cell wall to protrude slightly (Fig. 18.1c) (Pham et al. 2011b). Taken together the synergistic effect of LiAc and ssDNA on transformation efficiency, we attributed the high transformation efficiency achieved with LiAc and ssDNA to this altered cell wall structure that was synergistically caused by LiAc and ssDNA (Pham et al. 2011b).

Using the natural transformation method (Hayama et al. 2002), we transformed approximately 5,000 strains in each of which a nonessential gene was deleted. Several deletion mutants had high transformation efficiency (e.g. *spf1*) whereas others had low transformation efficiency (e.g. *arc18* and *she4*), and the findings provided evidence that DNA enters the cell via endocytotic membrane invagination (Kawai et al. 2004). Using fluorescence microscopy, we visualized the process of transformation achieved by the

Table 18.1 ssDNA and LiAc synergistically enhance transformation efficiency and frequency

	Transformation efficiency cfu/μg		Viable cells cfu		Transformation efficiency (A/B)
Composition[a]	pRS415 (A)	Fold	(× 10^4) (B)	Fold	Fold
None[b]	2,008 ± 1,606	1	1,592 ± 8	1.00	1
ssDNA[c]	13,613 ± 8,226	7	1,821 ± 141	1.14	6
LiAc[d]	84,888 ± 37,692	42	1,128 ± 400	0.71	60
LiAc + ssDNA[e]	1,007,500 ± 657,319	501	1,428 ± 76	0.90	560

[a]The cells were incubated with 36 % PEG and 0.2 μg pRS415 at 42 °C for 20 min in 42 μl transformation mix containing no additional reagents[b], 0.29 mg/ml ssDNA[c], 10.7 mM LiAc[d], or both 10.7 mM LiAc and 0.29 mg/ml ssDNA[e]
(From Pham, T.A., S. Kawai, and K. Murata. 2011b. Visualization of the synergistic effect of lithium acetate and single-stranded carrier DNA on *Saccharomyces cerevisiae* transformation. Curr. Genet. 57: 233-239 with permission.)

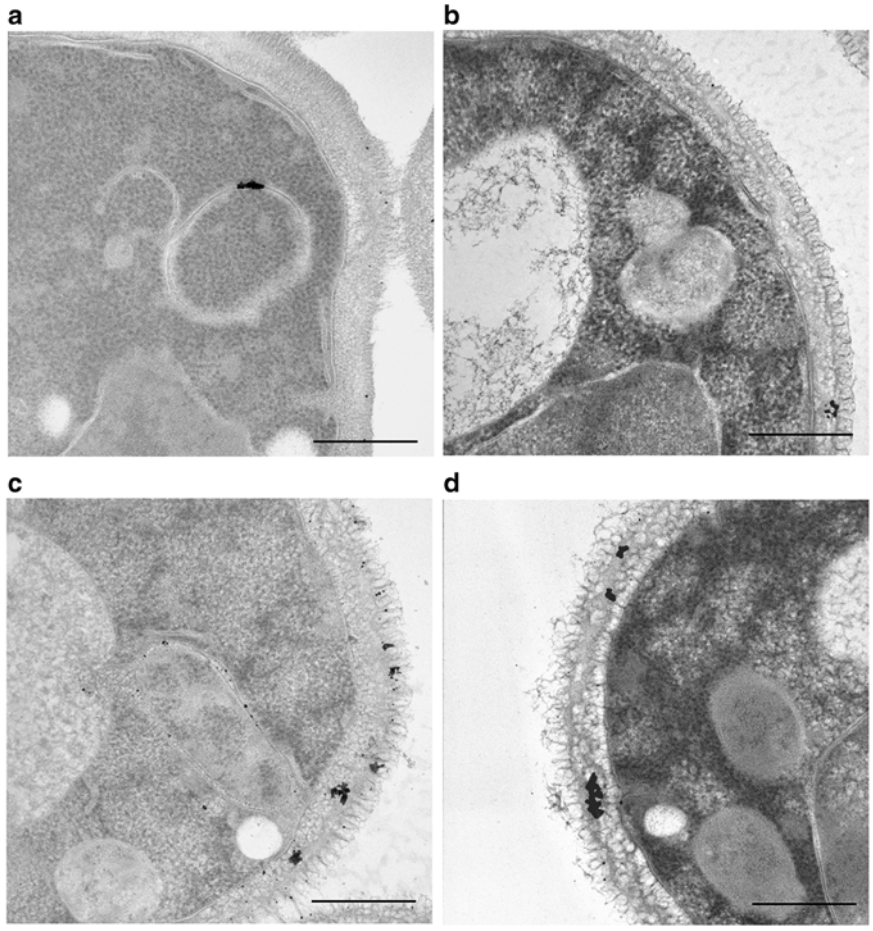

Fig. 18.1 Visualizing the effects of ssDNA and LiAc. Cells were incubated at 42 °C for 20 min with PEG and negatively charged Nanogold, in the presence of no additional reagents (**a**), ssDNA (**b**), LiAc (**c**), both LiAc and ssDNA (**d**) as described in Table 18.1, treated, and observed by transmission electron microscopy. Panels show images at 47,800-fold magnification (scale bar is 0.50 μm). Signals from Nanogold are observed as dots. For more detail, see the reference (From Pham, T.A., S. Kawai, and K. Murata. 2011b. Visualization of the synergistic effect of lithium acetate and single-stranded carrier DNA on *Saccharomyces cerevisiae* transformation. Curr. Genet. 57: 233-239 with permission.)

natural transformation method with YOYO-1-labelled plasmid DNA (YOYO-1/YEp13) (Pham et al. 2011a). YOYO-1 is a widely used cell-impermeable fluorescent DNA probe (Gurrieri et al. 1997). We observed that YOYO-1/YEp13 attaches to the region around intact cells incubated with PEG, and PEG was required for the attachment of YOYO-1/YEp13 onto cells and their successful transformation. Moreover, the fluorescence intensity of *spf1* cells was higher than that of wild type (WT) cells, and the intensity of unwashed cells was much higher than that of washed cells (Fig. 18.2). The transformation efficiency of unwashed cells was 14.8-fold (*spf1* cell) and 2.3-fold (WT cell) greater than that of washed cells, suggesting that washing the cells removes attached DNA from the cells and thereby decreases transformation efficiency. Based on these observations, we concluded that (i) the DNA absorbed on the cell surface is taken up by the cell; (ii) delivery of DNA into the nucleus mainly occur in cells spread on solid selective medium, and (iii) the high capacity of *spf1* cells to absorb DNA is at least partially responsible for

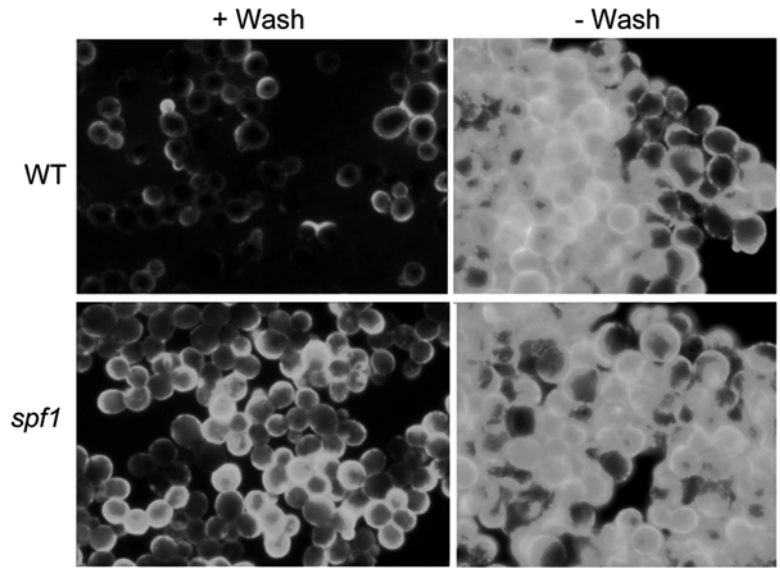

Fig. 18.2 Behaviour of YOYO-1/YEp13 during transformation. Intact WT (*upper*) and *spf1* (*lower*) cells were incubated at 30 °C for 1 h in 80 µl suspension containing 34 % (w/v) PEG and YOYO-1/YEp13 equivalent to 1.8 µg YEp13. The incubated cells were observed after washing (*left*: + Wash) or without washing (*right*: - Wash). (Note: LiAc and ssDNA were not included in the mixture). (From Pham, T.A., S. Kawai, E. Kono, and K. Murata. 2011a. The role of cell wall revealed by the visualization of *Saccharomyces cerevisiae* transformation. Curr. Microbiol. 62: 956-961 with permission.)

the high-transformation phenotype of *spf1* cells (Kawai et al. 2004, Pham et al. 2011a).

18.1.4 The Modified Lithium Method (LiAc/ssDNA/PEG Method)

We have recognized the high efficiency of LiAc/ssDNA/PEG method, and are now using a method that is essentially the same as the reported one (Gietz and Woods 2002). Below, we describe the practical procedure and provide some additional comments.

18.2 Materials

1. Pure water: prepared using an Elix Advantage 3 (Millipore). All reagents and media are prepared using pure water. Sterilized distilled water (SDW) is prepared by autoclaving (121 °C, 20 min).
2. Liquid YPD medium: 1.0 % yeast extract, 2.0 % tryptone, and 2.0 % glucose in pure water (pH 5.6). For solid medium, add 2.0 % agar. Sterilize medium by autoclaving (121 °C, 20 min). The antibiotic stock solution (e.g. geneticin, 50 mg/ml in pure water; hygromycin B, 50 mg/ml in pure water) is sterilized by filtration (Advantec, Dismic-25cs, Cellulose Acetate, pore size 0.20 µm) and added to YPD medium after autoclaving (final concentrations: geneticin, 100 µg/ml; hygromycin B, 300 µg/ml).
3. Synthetic complete (SC) medium: 0.67 % yeast nitrogen base without amino acids (Becton, Dickinson and Company), 2.0 % glucose, 690 mg/l-Leu Do supplement (Clontech), 100 mg/l leucine. For SC medium without nutrients, use the appropriate Do supplement (Clontech). Liquid medium is solidified by addition of 2 % agar.
4. 50 % PEG: Add 10 g PEG (Sigma, P-3640), 200 µl 1.0 M Tris-HCl (pH 8.0), 100 µl

200 mM EDTA (pH 8.0) to pure water. Dissolve the PEG, and add pure water to a final volume of 20 ml. Sterilize by autoclaving. Store at room temperature.

5. 1.0 M LiAc: Dissolve LiAc (Nacalai Tesque, Extra pure grade) in pure water to reach 1.0 M; pH is not adjusted. Sterilize by autoclaving. Store at room temperature.
6. TE: 10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA (pH 8.0), in pure water. Sterilize by autoclaving.
7. 2.0 mg/ml ssDNA: Dissolve salmon sperm DNA (Sigma, D-1626) in TE at 2.0 mg/ml with gentle stirring at room temperature for 2–3 h. Store at −30 °C. Just before use, thaw a small portion, incubate the aliquot in boiling water for 5 min, and immediately cool in ice/water. After the transformation, discard any remaining boiled and cooled ssDNA.
8. Transformation mix: Mix 240 µl 50 % PEG, 36 µl 1.0 M LiAc, and 50 µl 2.0 mg/ml ssDNA.

18.3 Methods

1. Inoculate the *S. cerevisiae* strain into 1.0 ml of liquid YPD medium, and cultivate the strain aerobically at 30 °C to reach saturation.
2. Transfer 30 µl of the saturated preculture to 1.5 ml liquid YPD medium and further cultivate the cells for 4–5 h.
3. Collect the cells by centrifugation at 14,000 *g* for 1 min. Remove the supernatant completely.
4. Add 40 µl of transformation mix to the cells, and resuspend the cells in the transformation mix by vigorous vortex mixing.
5. Add less than 4 µl of plasmid DNA (or DNA fragment) and mix by vigorous vortex mixing. *Comments*: PCR reaction mixture can be used without purification.
6. Incubate the tubes in a 42 °C water bath for 40 min.
7. For selective solid SC medium without nutrients: Place 100 µl SDW on solid selective medium. Collect the cells in the suspension by centrifugation at 14,000 *g* for 1 min, and discard the supernatant completely. Resuspend the cells in SDW (44 µl). Put the 2 µl suspension and the remaining suspension (−42 µl) onto 100 µl SDW on the solid selective media (Mix the suspension with the 100 µl SDW on the solid media). Spread the cells onto the solid media. When counting viable cells, add SDW to the cell suspension to reach 1.0 ml, suspend very gently by pipetting, dilute the suspension in SDW, and spread the dilutions onto solid YPD medium to count cells. With the remaining suspension, spread 50 µl onto the selective solid medium; then, collect the cells in the remaining suspension by centrifugation at 14,000 *g* for 1 min, and discard the supernatant. Resuspend the cells in SDW (less than 100 µl) and spread on the selective solid medium. *Comments*: If washing of cells is required to remove PEG, wash gently to avoid removing the DNA attached to the cells (Fig. 18.2).
8. For selective solid YPD medium with antibiotics: Collect the cells by centrifugation at 14,000 *g* for 1 min, and completely remove the supernatant. Suspend the cells in 1.0 ml SC liquid medium and cultivate the cells aerobically overnight at 30 °C. Spread 100 µl of the culture on the selective solid medium. Collect the cells in the remaining culture by centrifugation at 14,000 *g* for 1 min, and discard the supernatant. Resuspend the cells in the SDW (less than 100 µl) and spread on the selective solid medium.
9. Incubate solid medium at 30 °C for 3–4 days.

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Transformation of Lithium Acetate-treated *Neurospora crassa*

19

John V. Paietta

19.1 Introduction

The genetic modification of filamentous fungi by introduction of exogenous DNA through transformation has become a well-developed molecular genetic tool. A number of alternative transformation protocols have been refined for their use in fungi (e.g., spheroplasting, electroporation, and lithium acetate treatment). Of particular interest in this chapter is the transformation technique using the treatment of cells with alkali cations that was first reported by Ito, et al. (1983) in *Saccharomyces cerevisiae*. The protocol provided a fast, simple, and effective means of transformation. Following its development in yeast, the use of lithium acetate treatment was adapted for the transformation of the filamentous fungus *Neurospora crassa* (Dhawale et al. 1984) and subsequently, as described below, a variety of other filamentous fungi.

As originally developed, the transformation of *N. crassa* using lithium acetated-treated germinating conidia (Dhawale et al. 1984) provided a rapid, easy to setup, and efficient alternative approach to the preparation and immediate use of spheroplasts derived from glusulase digestion (Case et al. 1979). The spheroplasting transformation protocol was later refined to yield a high transformation frequency by using Novozym 234 and to allow for stable spheroplast storage at −80 °C with a cryoprotectant (Vollmer and Yanofsky 1986). Still, the lithium acetate protocol remains a convenient method to obtain *N. crassa* transformants for a variety of purposes. A small culture tube slant typically provides sufficient conidia for a transformation. Thus, the approach can be quickly performed simultaneously on a variety of strains, using mini-preps of DNA (Paietta and Marzluf 1984), without extensive optimization if only a relatively modest transformation frequency is needed. As an example, the potential range of application for lithium acetate transformation is demonstrated by its usage to generate targeted gene disruption/replacements at the *am*$^+$ locus (Paietta and Marzluf 1985). Such gene targeting experiments in *N. crassa* represented a low-probability event prior to the use of nonhomologous end-joining defective mutants as transformation host strains (Ninomiya et al. 2004).

Besides *N. crassa*, the lithium acetate protocol has been adapted successfully in a variety of filamentous fungal species in the Ascomycota and Basidiomycota (see Table 19.1). The list of species includes a number of plant and animal pathogens as well as those of biotechnological importance. In some cases, the use of transformation by lithium acetate treatment has been

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Table 19.1 Examples of dimorphic and filamentous fungal transformation using lithium acetate

Fungal Species	Reference
Ascomycota:	
Colletotrichum capsici	Soliday et al. (1989)
Colletotrichum trifoli	Dickman (1988)
Fusarium graminearum	Dickman and Partridge (1989)
Fusarium moniliforme	Dickman and Partridge (1989)
Fusarium solani (f. sp. *pisi* and f. sp. *phaseoli*)	Soliday et al. (1989); Marek et al. (1989)
Histoplasma capsulatum	Worsham and Goldman (1990)
Humicola grisea var. *thermoidea*	Allison et al. (1992); Danta-Barbosa et al. (1998)
Mycosphaerella spp.	Dickman et al. (1989)
Neurospora crassa	Dhawale et al. (1984)
Basidiomycota:	
Coprinus cinereus	Binninger et al. (1987)
Ustilago maydis	Butters and Hollomon (1996)
Ustilago violacea	Bej and Perlin (1989)

reported to be the preferred protocol where application or optimization of a spheroplasting approach is difficult. Presented here is the basic *N. crassa* protocol with changes made to adapt the lithium acetate technique to other species noted.

19.2 Materials

19.2.1 Solutions and Reagents for *N. crassa* Culturing

1. Biotin solution: 2.5 mg biotin in 50 ml of 50 % (v/v) ethanol.
2. Trace elements solution: 5 g $C_6H_8O_7 \cdot H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 1 g $Fe(NH_4)_2(SO_4) \cdot 6H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.05 g $MnSO_4 \cdot H_2O$, 0.05 g H_3BO_3, and 0.05 g $NaMoO_4 \cdot 2H_2O$ in 100 ml. Add 1 ml chloroform as preservative. Store at 4 °C.
3. Fries medium: per liter, dissolve 5.0 g $(NH_4)_2$ tartrate; 1.0 g NH_4NO_3, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.1 g NaCl, 0.1 ml biotin solution, and 0.1 ml trace elements solution (Davis and deSerres 1970).
4. Vogel's salts (50x): per liter, 126.8 g $Na_3C_6H_5O_7 \cdot 2H_2O$, 250 g KH_2PO_4, 100 g NH_4NO_3, 10 g $MgSO_4 \cdot 7H_2O$, 5 g $CaCl_2 \cdot 2H_2O$, 5 ml Biotin solution, and 5 ml trace elements solution. Chloroform (5 ml) added as preservative (Davis and deSerres 1970).
5. Sorbose solution (for induction of colonial growth): 10x solution is per 500 ml; 100 g L-sorbose, 2.5 g fructose, and 2.5 g glucose. Autoclave and store at room temperature.
6. Hygromycin B (Calbiochem)
7. Benomyl (DuPont or Sigma)

19.2.2 Solutions for Lithium Acetate Transformation

Solutions are autoclaved and stored at room temperature.

1. 0.1 M lithium acetate: Dissolve 5.1 g of lithium acetate dihydrate in 100 ml of water.
2. TE: 10 mM Tris, 1 mM EDTA, pH 7.5
3. 40 % PEG 3350 in 0.1 M lithium acetate: 40 g PEG 3350 (Sigma P4388) dissolved in sufficient 0.1 M lithium acetate to yield 100 ml total volume.

19.3 Methods

19.3.1 Lithium Acetate Transformation

1. *N. crassa* cultures are typically grown on slants of Fries or 1x Vogel's medium with 1.5 % sucrose as a carbon source. Conidia from 7–8-day-old cultures are harvested by suspending in sterile water and filtering through cheesecloth. Following centrifugation (3 min at 1,000 × *g*; Damon HN-S centrifuge: swinging bucket rotor), conidia are resuspended in 0.5x Fries (or 0.5x Vogel's) medium with 0.75 % sucrose and with supplements as needed. The conidial preparation is incubated for 2.5 h at 30° C at 100 rpm in an orbital shaker. Up to 1×10^8 conidia per 150 ml can be used.

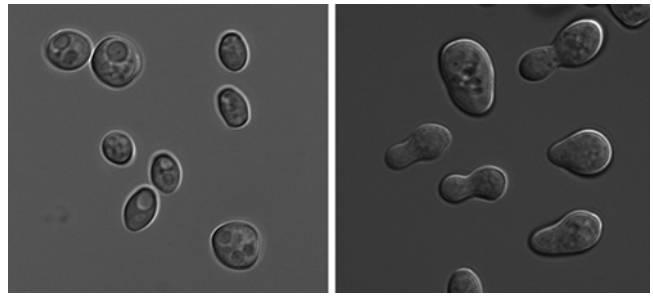

Fig. 19.1 Conidial preparations for lithium acetate transformation. Shown are: (*left panel*) freshly harvested *Neurospora crassa* conidia and (*right panel*) germinating conidia after a 2.5 h incubation that are ready for use in the lithium acetate transformation protocol. Note the early stage of germ tube formation after the initial incubation period which yields an optimal frequency of transformation using lithium acetate. DIC (differential interference contrast) micrograph images were obtained with a Zeiss Observer. D1 using a 60x oil immersion objective

2. The germinating conidia (Fig. 19.1) are sedimented by centrifugation in sterile 15 ml conical polystyrene tubes (Corning #25311) at 1,000 × *g* for 3 min. Each tube of conidia is washed in 10 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, and sedimented by centrifugation as above.
3. The washed conidia are resuspended in 10 ml of 0.1 M lithium acetate in the same 15 ml conical centrifuge tube. They are shaken gently (100 rpm) for 30 min at 30 °C in water bath shaker.
4. The lithium acetate-treated conidia are pelleted by centrifugation, as above, and resuspended in 0.4 ml of 0.1 M lithium acetate in the same centrifuge tube.
5. Up to 20 µg of plasmid DNA is added[1,2] and the mixture is gently shaken at 30 °C for 30 min. (5×10^7 conidia are typically used in 0.4 ml volume of lithium acetate).
6. 4 ml of 40 % PEG in 0.1 M lithium acetate is added to the DNA/conidial preparation. Incubation is continued with gentle shaking for 1 h at 30 °C. Some settlement of the conidia will occur during the incubation.
7. The sample is then heat shocked for 5 min at 37 °C.
8. The lithium acetate technique has been successfully adapted to a variety of fungi by modifying a number of parameters compared to the *N. crassa* protocol as presented here (see Table 19.1 for a compilation of species and references). Examples of species-dependent modifications, relative to the *N. crassa* protocol, to optimize lithium acetate transformation include: (1) extended incubation times for germination up to 48 h (e.g., *Coprinus cinerus*; Binninger et al. 1987), (2) adjustment to incubation and heat shock temperatures (e.g., *Humicola grisea*; Allison et al. 1992, Danta-Barbosa et al. 1998), (3) use of spermidine and spermine (e.g., *Colletotrichum trifolii*; Dickman 1988), (4) extension of the PEG treatment time (e.g., *Fusarium solani*; Soliday et al. 1989), and (5) an initial incubation of directly plated cells on nonselective medium followed by the addition of an overlay of agar containing the selective agent (e.g., *Fusarium solani*; Soliday et al. 1989).

19.3.2 Screening or Selection of Transformants

1. Direct plating of the heat shocked conidia is effective when using, for example, an auxotrophic mutant (e.g., *arom-9 qa-2*) (Case et al. 1979) as the host and using a transforming vector containing the wild-type gene (e.g., *qa-2*$^+$) gene with selection for prototrophy. For these applications, the heat shocked conidia are centrifuged (as above); washed once with water, pelleted a final time, and resuspended in water. The washed conidia are

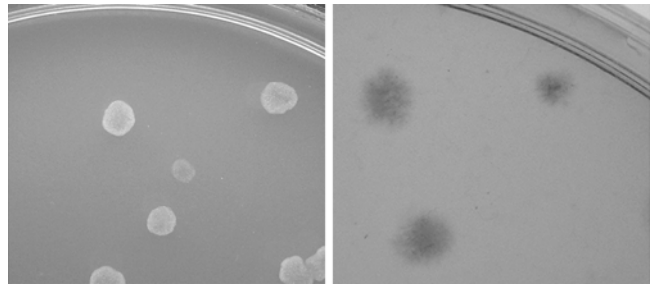

Fig. 19.2 Typical appearance of *Neurospora crassa* transformants following incubation of a direct (surface) plating using an auxotrophic to prototrophic selection (*left panel*) or overlaying the transformed conidia onto hygromycin B containing medium (*right panel*). Individual colonies within the overlay are transferred to agar slants containing the selection medium using a sterile microspatula

spread directly on the surface of the petri plates on Fries or 1x Vogel's medium containing 1x sorbose solution. Plates are incubated at 30 °C with transformants appearing in 4–5 days depending on the strains used (Fig. 19.2). Typically 1x sorbose solution is added to ensure colonial growth and less background growth. As an example, 20 μg of *qa-2*$^{+}$ plasmid DNA (e.g., pVK57) (Alton et al. 1978) should yield approximately 400 transformants with this protocol, i.e., 20 transformants per microgram DNA.

2. For drug resistance selection, higher transformation frequencies with less background growth are obtained using a bottom agar layer containing the selective compound followed by overlaying top agar (typically nonselective) with the transformed conidia. In this case, following heat shock the conidial mixture still containing the PEG, can be added directly to top agar (cooled to 50 °C) containing Fries or 1x Vogel's with sorbose solution and poured over the drug underlay. Under such conditions hygromycin B is generally used in the bottom layer at a concentration of 200 μg/ml and benomyl at 0.5 μg/ml (Orbach et al. 1986, Staben et al. 1989).
3. Typically, most *N. crassa* transformants will be heterokaryotic and in many cases it will be desirable to work with homokaryons. The growth of primary transformants on iodoacetate followed by passage of harvested conidia through a 5 μm filter provides a convenient means of isolating homokaryotic microconidia (Ebbole and Sachs 1990). The isolation and plating of microconidia provides an efficient alternative to repeated streaking and re-isolation of transformants to ensure the homokaryotic nature of the isolate to be studied.

19.4 Notes

1. As reported initially (Dhawale et al. 1984), pretreatment of DNA with heparin as is commonly done for spheroplasting reduces the frequency of transformation in *N. crassa* using lithium acetate.
2. In *Saccharomyces cerevisiae*, addition of single-stranded carrier DNA leads to a marked improvement in transformation efficiency (Gietz and Schiestl 2007). Trial modifications of the *N. crassa* protocol to include single-stranded carrier DNA have not resulted in improvements of transformation frequency (Paietta, unpublished data).

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Part VII

Transformation Methods: New Developments

Application of Novel Polymeric Carrier of Plasmid DNA for Transformation of Yeast Cells

20

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20.1 Introduction

Yeasts are widely used as efficient producers of various biologically active substances in industrial biotechnology, as well as experimental models of signaling processes taking place in higher eukaryotic cells, and a powerful model system for unveiling mechanisms of molecular pathogenesis of numerous human diseases. DNA delivery into cells is key in the development of new, efficient yeast strains and yeast-based studies. Various approaches have been developed for delivery of DNA into yeast cells, including a lithium acetate (Li/Ac) method, electroporation, two-hybrid system transformation, biolistic transformation, protoplast transformation, and others (Armaleo et al. 1990; Becker and Guarente 1991; Bartel and Fields 1995; Butow et al. 1996; Gietz and Woods 2001; Kawakami et al. 2006; Brzobohaty and Kovac 1996). Efficient yeast transformation remains a challenging task because yeast cells are surrounded by a thick wall composed mostly of mannose-containing proteins and glycans (Zlotnik et al. 1984). Typical protocols for DNA delivery into yeast cells include time consuming and/or expensive enzymatic removal of cell wall with lyticase or zymolyase; as well as chemical pretreatment of yeast cells with polyethylene glycol, lithium chloride, or thiol compounds like 2-mercaptoethanol, dimethyl sulfoxide (DMSO), or dithiothreitol (DTT) making the cell wall leaky to macromolecules (Reddy and Maley 1993; Ito et al. 1983a, b; Schiestl et al. 1993; Hill et al. 1991). A significant progress was achieved in the development of new approaches of yeast transformation by using various particles for delivery of plasmid DNA (Kawakami et al. 2006; Zhong et al. 2010; Polu and Kumar 2011). Transformation of yeast cells was demonstrated by their agitation with glass beads (0.3 g, diameter 0.45–0.52 mm) in the presence of plasmid DNA (Costanzo and Fox 1988). "Gene gun" was the most effective for transformation of specific yeast cells refractory to DNA engulfment. Yeast cells were bombarded with 0.5 mm gold or tungsten DNA-coated "projectiles" using compressed helium. However, the effectiveness of this technique strongly depends on cellular characteristics (composition of the cell wall), and application of this method requires expensive equipment, it is time consuming, and

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still provides relatively low efficiency of yeast transformation (Bartel and Fields 1995; Butow et al. 1996).

Polyethylene glycol is the best presently known polymeric agent widely used in yeast transformation. However, this agent is used only for transformation of yeast protoplasts or spheroplasts. Besides, it is a time consuming and complicated method with irreproducible and often unsatisfactory results (Hill et al. 1991; Gietz and Woods 2002; Klebe et al. 1983). Significant progress in the development of yeast transformation was achieved by using specific nanoscale particles for DNA delivery (Butow et al. 1996; Gietz and Woods 2001; Brzobohaty and Kovac 1996).

The two most often applied methods of DNA delivery into yeast cells—electroporation and the Li/Ac method—are not only time consuming, but also not efficient for some yeast species of high biotechnological interest (Armaleo et al. 1990; Gietz and Woods 2001; Brzobohaty and Kovac 1996). *Yarrowia lipolytica*, *Dekkera bruxellensis* (*Brettanomyces bruxellensis*), *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*), *Hansenula polymorpha*, and *Candida lipolytica* are yeast species possessing both high potential in biotechnology and drawbacks in their transformation by heterologous DNA. These species have been shown to be potential producers of bioethanol, biogas, or other biological products of industrial interest.

Thus, the lack of convenient, efficient, and nontoxic method for DNA delivery remains one of the biggest challenges in yeast biotechnology and basic research. Here we propose a novel transformation method for plasmid DNA delivery into the yeast cells. It is based on using a new nanoscale comb-like oligoelectrolyte polymer which combines an anionic backbone and dimethyl aminoethyl methacrylate (DMAEM)-based side branches for DNA delivery into yeast cells of several species.

20.2 Materials

1. Yeast cells of different strains
2. Plasmid DNA
3. Liquid **YPD** medium (1 % **y**east extract, 2 % bacto-**p**eptone, 2 % **D**-glucose)
4. Selective YPD medium supplemented with antibiotic G418 (Geneticin) (50 mg/L) (Invitrogen, Sweden) or other selective medium
5. Polymeric oligoelectrolyte carrier of plasmid DNA
6. Thermostat for yeast incubation
7. Spectrophotometer
8. Centrifuge
9. Eppendorf microcentrifuge (5415D)
10. Water bath
11. Petri dishes
12. 1.5 mL microcentrifuge tubes or 1.5 mL Eppendorf tubes

The novel carrier of plasmid DNA named BG-2 in our laboratory is a copolymer of a comb-like structure that combines an oligoelectrolyte chain of the anionic type, as a backbone, with 1–3 grafted side chains of the cationic type (Fig. 20.1). Combination of these chains provides the carrier molecule with optimal surface activity and controlled solubility in a wide pH range. Besides, these molecules possess an ability to form inter-oligoelectrolyte complexes in the water-based systems.

This oligoelectrolyte polymer was synthesized by a controlled radical polymerization initiated by the oligoperoxide metal complex (OMC) in a polar organic media. OMC was coordinating Cu^{2+} complex of the copolymer composed of vinyl acetate (VA), 5-tertbutylperoxy-5-methyl-1-hexene-3-yne (VEP), and maleic anhydride (MA). Both the initial oligoperoxide and OMC derivate have been synthesized, as described (Zaichenko et al. 1997, 2000, 2001). Principal characteristics of the resulting BG-2 oligoelectrolyte carrier are presented in Table 20.1.

Synthesized polymer was dissolved in sterile distilled H_2O at 1 %, pH 7.4 (if not mentioned otherwise) and stored at 4 °C in 1.5 mL Eppendorf tubes leaving as less air in the tube as possible.

20.3 Methods

The method presented below describes procedures for enabling easy and effective delivery of plasmid DNA into yeast species. Modifications may

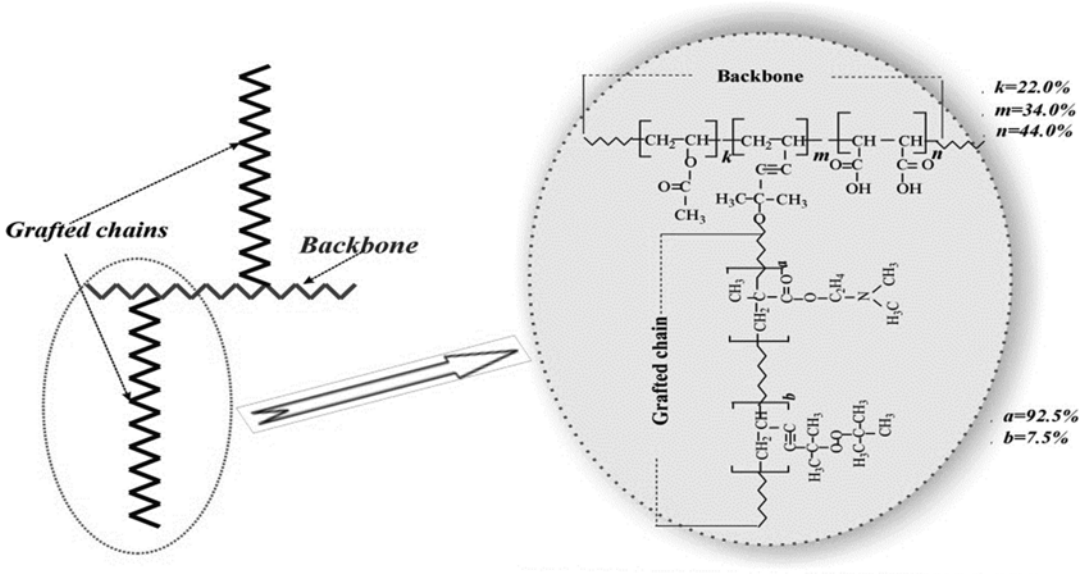

Fig. 20.1 Schematic structure of the BG-2 polymeric carrier (a, b, k, m, n—see Table 20.1) (From Filyak, Ye. and N. Finiuk, N. Mitina, O. Bilyk, V. Titorenko, O. Hrydzhuk, A. Zaichenko, and R. Stoika. 2013. A novel method for genetic transformation of yeast cells using oligoelectrolyte polymeric nanoscale carriers. BioTechniques. 54: 35–43 with permission)

Table 20.1 Principal characteristics of the polyelectrolyte carrier BG-2

	Backbone								Grafted chains					
	Content of monomer links in oligoelectrolyte (%)			Characteristics of backbone microstructure				[Cu^{2+}] (% per main chain)	Content of monomer links in oligo-electrolyte (%)		Characteristics of microstructure			Surface tension (mN/m)
	VA *k*	VEP *l*	MA *m*	l_{VA}	l_{VEP}	l_{MA}	R		VEP *b*	DMAEM *a*	l_{VEP}	l_{DMAEM}	R	
BG-2	22.0	34.0	44.0	1.0	1.0	1.0	99.5	1.1	7.5	92.5	1.02	14.8	7.5	31.5

l—average length of the blocks from the corresponding monomer links, R—average amount of the blocks from the same monomer links per 100 links in the copolymer (From Filyak, Ye. and N. Finiuk, N. Mitina, O. Bilyk, V. Titorenko, O. Hrydzhuk, A. Zaichenko, and R. Stoika. 2013. A novel method for genetic transformation of yeast cells using oligoelectrolyte polymeric nanoscale carriers. BioTechniques. 54: 35–43 with permission)

be needed to optimize this transformation method for other yeasts species (see Notes).

1. Grow yeasts in nonselective YPD medium at 37 °C (*H. polymorpha*) or 30 °C (*P. pastoris*, *S. cerevisiae*) overnight.
2. Transfer 150 μL of the overnight yeast culture into 30 mL of YPD medium.
3. Grow suspension of yeast cells on shaker at 37 °C or 30 °C until OD_{600} 0.5–0.7 (approximately 2 h).
4. Transfer suspension of yeast cells into 1.5 mL Eppendorf microcentrifuge tubes.
5. Collect the yeast cells by centrifugation (10 min at 3,000 g).
6. Resuspend yeast cells in 100 μL of YPD in a microcentrifuge tube.
7. Prepare the transformation mixture: add 1 μL of 1 % solution of oligoelectrolyte-based carrier BG-2 (adjusted to pH 7.4 with 1 M NaOH) and 1 μg of plasmid DNA to a microcentrifuge tube.
8. Add 15 μL of 1 M $CaCl_2$.
9. Add the transformation mixture to the yeast cell suspension (100 μL).

10. Mix the suspension gently and keep on ice for 45 min.
11. Heat shock yeast cells for 60–90 s at 42 °C (*H. polymorpha*) or 55 °C (*P. pastoris*, *S. cerevisiae*).
12. Chill yeast cells on wet ice for 2 min.
13. Mix yeast cells with 1 mL of YPD medium.
14. Incubate yeast cells for 1 h at 37 °C (*H. polymorpha*) or 30 °C (*P. pastoris*, *S. cerevisiae*).
15. Plate 100 μL of yeast cells on a selective medium (see item 8.2.4).
16. Incubate yeast cells at 37 °C (*H. polymorpha*) or 30 °C (*P. pastoris*, *S. cerevisiae*).
17. Count yeast transformants after 3–5 days of cultivation.

20.4 Notes

Optimization of the transformation protocol for specific yeast strains may be needed when other species are used. If necessary, the pH of the transformation solution as well as other conditions could be changed, (e.g., plasmid DNA ratio: oligoelectrolyte-based carrier, heat shock temperature, concentration of $CaCl_2$).

20.5 Results

H. polymorpha is a popular lower eukaryotic organism to study methanol metabolism, peroxisome biogenesis and degradation, biochemistry of nitrate assimilation, resistance to toxic metals and oxidative stress, as well as production of recombinant proteins and commercial pharmaceuticals (Dmytruk et al. 2007; Faber et al. 1992; Smutok et al. 2007).

We compared the results of transforming *H. polymorpha* NCYC 495 leu1-1 yeast by a circular plasmid pGLG578 (carrying *LEU2* of *S. cerevisiae* as a selectable marker) or linear *Hin*dIII-digested plasmid pYT3 (also carrying Sc*LEU2*) (Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine) either using the novel BG-2 polymer-based transformation method, traditional LiAc method (Ito et al. 1983b), and electroporation (Becker and Guarente 1991). Leu^+ transformants were selected on the minimal modified Burkholder medium (Dmytruk et al. 2007) without Leucine, while geneticin® (G418) resistant transformants were selected on YPD media supplemented with G418 (50 mg/L) using pGLG578 plasmid. When pYT3 was used, Leu^+ transformants were selected on a solid minimal modified Burkholder media without Leucine described in (Dmytruk et al. 2007).

A developed transformation protocol resulted in twofold increase in transformants (using linear plasmid pYT3) than at using electroporation, and 15.7-fold increase compared to LiAc method (Fig. 20.2). Delivery of nonlinearized plasmid DNA pGLG578 with BG-2 carrier resulted in two times more *H. polymorpha* transformants than with electroporation, and 62 times more transformants than with LiAc method (Fig. 20.3).

P. pastoris yeast is frequently used as an expression system for production of heterologous proteins (Scharstuhl et al. 2003; Razaonov and Strongin 2003; Daly and Hearn 2005). His^+ transformants of *P. pastoris* were selected on a solid minimal modified Burkholder medium (Dmytruk et al. 2007) without Histidine.

Application of BG-2-based method for genetic transformation of *P. pastoris* yeast with linearized pPIC3.5 carrying *HIS4* as a selective marker (Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine) resulted in five times more transformants compared with using electroporation, and 79 times more transformants compared with using LiAc method (Fig. 20.4).

The novel carrier was also applied for genetic transformation of *S. cerevisiae* yeast. However, its efficiency was not increased when BG-2 was used comparing with the transformation indicators of application of lithium acetate or electroporation methods. While, as noted above, the indicator of the BG-2-based transformation of the *H. polymorpha* and *P. pastoris yeasts* were significantly higher than the transformation efficiency when lithium acetate or electroporation methods were utilized.

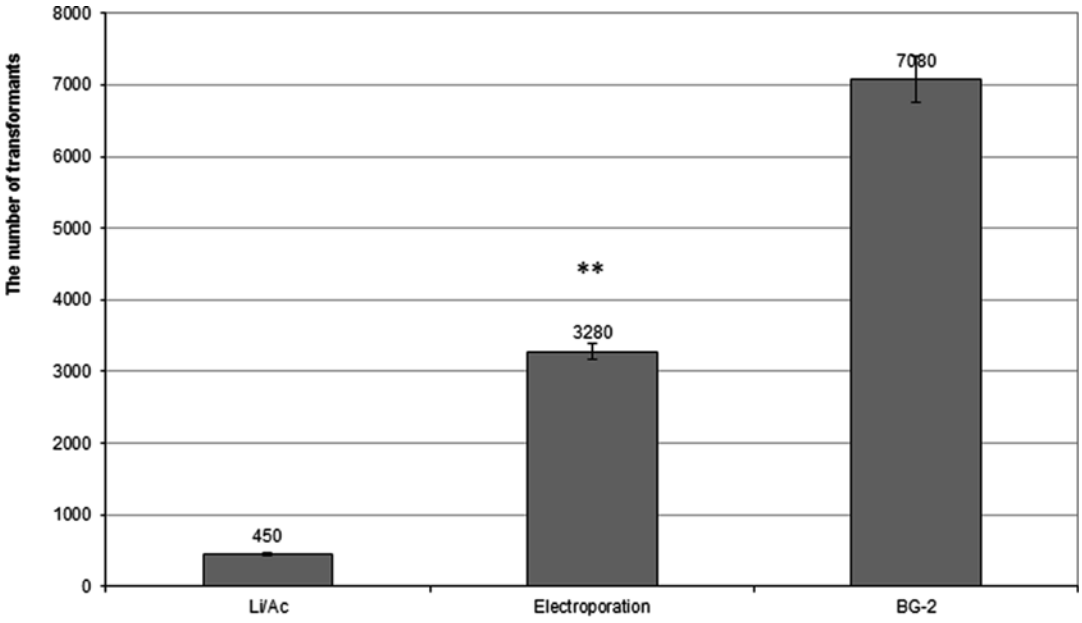

Fig. 20.2 Number of *H. polymorpha* NCYC 495 leu1-1 yeast transformants obtained by using different transformation methods. Lithium acetate, electroporation, and the BG-2 carrier-based method were compared using the linearized pYT3 plasmid, $^{**}P<0.01$ (From Filyak, Ye. and N. Finiuk, N. Mitina, O. Bilyk, V. Titorenko, O. Hrydzhuk, A. Zaichenko, and R. Stoika. 2013. A novel method for genetic transformation of yeast cells using oligoelectrolyte polymeric nanoscale carriers. BioTechniques. 54: 35–43 with permission)

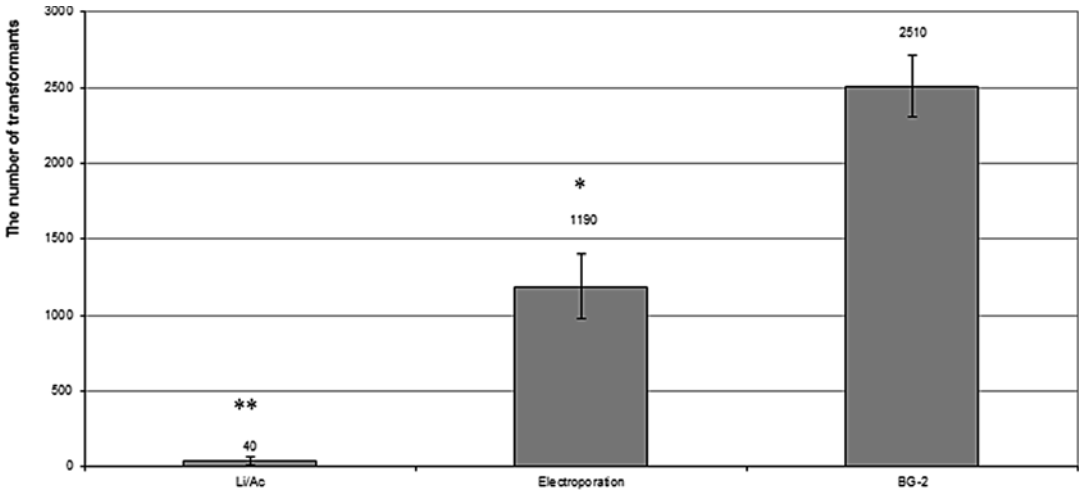

Fig. 20.3 Number of *H. polymorpha* NCYC 495 leu1-1 yeast transformants obtained through different transformation methods. Lithium acetate, electroporation, and the BG-2 carrier-based transformation method were compared using the circular plasmid pGLG578. Yeasts were selected on G418, $^{*}P<0.05$, $^{**}P<0.01$ (From Filyak, Ye. and N. Finiuk, N. Mitina, O. Bilyk, V. Titorenko, O. Hrydzhuk, A. Zaichenko, and R. Stoika. 2013. A novel method for genetic transformation of yeast cells using oligoelectrolyte polymeric nanoscale carriers. BioTechniques. 54: 35–43 with permission)

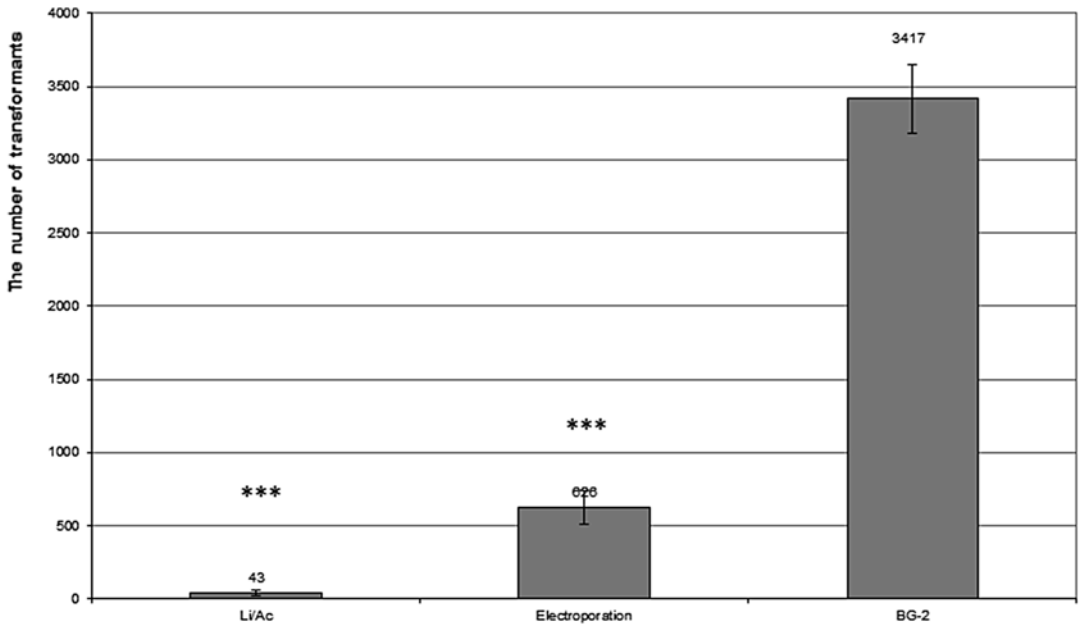

Fig. 20.4 Number of *P. pastoris* GS115 his4 yeast transformants obtained by using different transformation methods. Lithium acetate method, electroporation, and BG-2 carrier-based transformation method were applied for genetic transformation with a linear pPIC3.5 plasmid. Yeast cells were plated on a solid Histidine-deficient selective medium, $***P<0.001$ (From Filyak, Ye. and N. Finiuk, N. Mitina, O. Bilyk, V. Titorenko, O. Hrydzhuk, A. Zaichenko, and R. Stoika. 2013. A novel method for genetic transformation of yeast cells using oligoelectrolyte polymeric nanoscale carriers. BioTechniques. 54: 35–43 with permission)

20.6 Conclusion

This method is based on transformation of various yeast species without additional treatment and preparation of competent cells. It is efficient for genetic transformation of the yeast. Besides, it is nontoxic and non-mutagenic, and gives more reproducible results of genetic transformation compared to LiAc method and electroporation. The developed polymeric carrier can form complexes with either linearized or circular plasmid DNA. The novel polymeric carrier exhibits low toxicity and is not mutagenic (Filyak et al. 2013). The developed method of yeast transformation is convenient and rapid when compared to existing methods, and it does not require any special equipment for conducting the transformation.

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Transformation of Fungi Using Shock Waves

21

Miguel A. Gómez-Lim, Denis Magaña Ortíz, Francisco Fernández, and Achim M. Loske

21.1 Introduction

The manipulation of metabolic pathways of the genome of any organism depends on the availability of efficient methods for introduction of foreign DNA in a stable form. Genetic transformation has become a key area of fungal research, not only for basic research but also for important biotechnological applications. Fungal genetic transformation dates back 40 years when the group of Tatum transformed *Neurospora crassa*, employing inositol as a selectable marker (Mishra and Tatum 1973). The authors reported that the procedure was not reproducible and that the mutants were unstable and might spontaneously revert to the wild type at a low but significant frequency. A critical development for the future of the procedure was made by Hutchinson and Hartwell (1967), who developed a way of preparing *Saccharomyces cerevisiae* protoplasts (or spheroplasts) by dissolving the cell wall with a commercial cocktail containing hydrolytic enzymes, such as 1,3 glucanase and chitinase (Glusulase). Hinnen et al. (1978) subsequently discovered that protoplasts prepared in this way could be readily transformed in the presence of calcium chloride and over the next several years the use of protoplasts for transformation was extended to other fungi, including filamentous fungi such as *N. crassa* and *Aspergillus nidulans* (Case et al. 1979; Tilburn et al. 1983). Protoplasts from filamentous fungi have been prepared from a variety of cell types such as macroconidia, microconidia, and young mycelium.

To date, the original protocols have been improved, but have not been fundamentally changed. Anecdotal evidence suggests that the particular batch of enzyme used is of great importance to obtain functional protoplasts, but the main drawback is that the enzyme cocktails for protoplast preparation are not well-defined.

Several methods were developed as alternatives to protoplasts such as the use of mutant strains with more permeable membranes or cell walls (Fincham 1989), the use of high concentrations of alkali metal ions to induce permeability to DNA in intact cells (Iimura et al. 1983) and encapsulation of DNA in liposomes and fusion with protoplasts (Radford et al. 1981). All of these methods were very inefficient (low transformant yields), non-reproducible, and in some cases the mutants were unstable. Since the introduction of novel genes and the manipulation of specific

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metabolic routes of fungi have had an increasing demand in several disciplines, other transformation methods have been developed. These include PEG-mediated protoplast fusion, electroporation, biolistic transformation, and *Agrobacterium*-mediated transformation (reviewed in other chapters of this book). Consequently, a number of fungal species have been transformed successfully (Ward 2012). However, just like before, all of these methods still suffer from several drawbacks such as low frequency of transformation and problems of reproducibility (Lorito et al. 1993; Ozeki et al. 1994; Ruiz-Diez 2002; Michielse et al. 2005; Su et al. 2012). Furthermore, current methods are not suitable for high throughput experimentation. This has hampered, for example, the generation of libraries of randomly tagged mutants and other similar developments. In addition, there are many species of fungi that have proved recalcitrant to transformation by any of these methods (Meyer 2008). We have developed a new method, based on the use of underwater shock waves that is highly efficient, quite simple (intact spores, conidia, or mycelia can be used), and widely applicable.

The ideal method for transformation of fungi should be highly efficient, applicable to all species, not dependent on one particular type of tissue, fast, simple, and cost-effective. This was the rationale for the development of a novel physical method for fungal transformation involving shock waves.

21.2 Theoretical Background

Shock waves have been used in medicine to disintegrate urinary stones, gallbladder stones, pancreatic duct stones, salivary stones, to treat the Peyronie's disease, coronary vessels, as well as diseases in orthopedics and traumatology (Thiel 2001; Loske 2007; Ueberle 2011). Most clinical devices are based on one of three shock wave generation modes: electrohydraulic, piezoelectric, or electromagnetic (Lingeman 2007; Loske 2007). Energy focusing is achieved by reflectors, acoustic lenses, or spherically curved sources, resulting in a compression pulse with a rise time

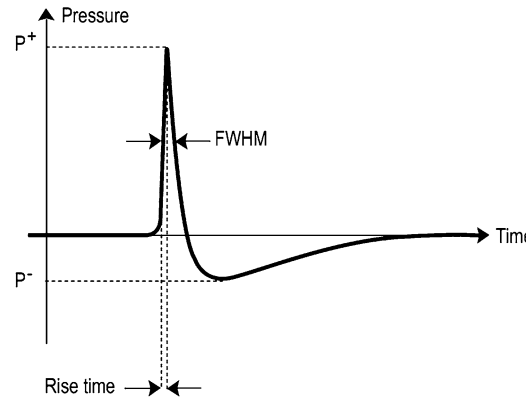

Fig. 21.1 Sketch of a pressure waveform recorded at the focus of a shock wave generator, showing the peak positive pressure p^+, the peak negative pressure p^-, the rise time and the full-width-half-maximum. The rise time was increased for clarity

of less than 10 ns and a peak positive pressure (p^+) of up to 150 MPa, followed by a tensile pulse (p^-) of up to 25 MPa, propagating through the medium at approximately 1,500 m/s (Fig. 21.1). The full-width-half-maximum, i.e., the time from the instant when the pressure first reaches 50 % of p^+ to the moment when it again falls to 50 % of p^+, is about 0.5 to 3 µs. The dynamic focus or focal region, defined as the volume in which, at any point, the positive pressure amplitude is equal to or higher than 50 % of p^+ can be imagined as an elliptical cigar shaped volume aligned along the axis of symmetry of the shock wave source. The shape and size of this volume varies depending on the design of the shock wave source and on the energy setting. Away from the dynamic focus, the pressure profile changes, having a significantly longer rise time and smaller p^+ and p^- values. Because of the possibilities for pressure profile shaping, piezoelectric shock wave generators are particularly suitable for applications in medicine and biotechnology.

In vivo and in vitro studies revealed that shock waves may cause transient cell permeabilization, allowing large molecules (normally excluded by the cell membrane) to become trapped inside the cell, opening the possibility of shock wave drug delivery and gene transfection (Gambihler et al. 1994; Lauer et al. 1997; Bao et al. 1998; Tschoep et al. 2001; Schaaf et al. 2003; Doukas and

Kollias 2004; Michel et al. 2004; Bekeredjian et al. 2007; Murata et al. 2007). Localized delivery of macromolecules into cells within living tissue (Kodama et al. 2000, 2002), as well as shock wave-mediated bacterial transformation has also been reported (Jagadeesh et al. 2004; Loske et al. 2011). As far as we know, the first study on underwater shock waves to transform filamentous fungi was recently published by our group (Magaña-Ortiz et al. 2013). Even if the detailed transformation mechanism is still unclear, it is known that acoustic cavitation is one of the main phenomena influencing cell membrane permeability.

Underwater shock waves can be coupled into a small fluid-filled polypropylene vial or bag centered at the focus of a shock wave source. Under normal conditions, a cell suspension will contain microbubbles and cavitation nuclei. At the focal point the positive pressure of the shock wave suddenly compresses each microbubble, enormously increasing the pressure inside it. This pressure and the trough following p^+ produce a rapid bubble growth. A few hundred microseconds later each bubble collapses violently, losing its spherical symmetry; leading to a fluid jet that pierces its way through the bubble, exiting at the other side at a velocity of up to 400 m/s (Philipp et al. 1993; Arora et al. 2005; Johnsen and Colonius 2008). Bubble collapse energy and microjet emission depend on the pressure profile (Canseco et al. 2011). According to Ohl and Ikink (2003), the microjets can act as syringes, injecting a volume of fluid of approximately 0.1 R^3 into the cells, where R is the bubble radius before arrival of the shock wave. Secondary shock waves are generated when the jet impacts the distal side of the bubble (see Fig. 21.2). At a shock wave generation rate of 1 Hz, bubbles produced by consecutive shock waves do not produce observable interference; however, nuclei seeded by cavitation may still exist as the next shock wave arrives. The pressure produced by the secondary shock waves can be extremely high; however, its effects are confined to very small dimensions (Brujan et al. 2008). Nevertheless, secondary shock waves may interact with other cavitation bubbles. The phenomenon is believed to be similar to sonoporation by ultrasound. In aqueous solutions, ultrasound forms bubbles, which create pores of approximately 30–100 nm in the bacterial membrane enabling uptake of molecules into the cell (Liu et al. 2006). The membrane recovers after a few seconds (Newman and Bettinger 2007).

It has been demonstrated that the energy of acoustic cavitation can be increased if a second shock wave is sent shortly before the bubble starts to collapse. These so-called "tandem" shock waves have been used to improve shock wave-induced transfer of DNA into bacteria (Loske et al. 2011) and to enhance genetic transformation of *A. niger* (Loske et al. 2014).

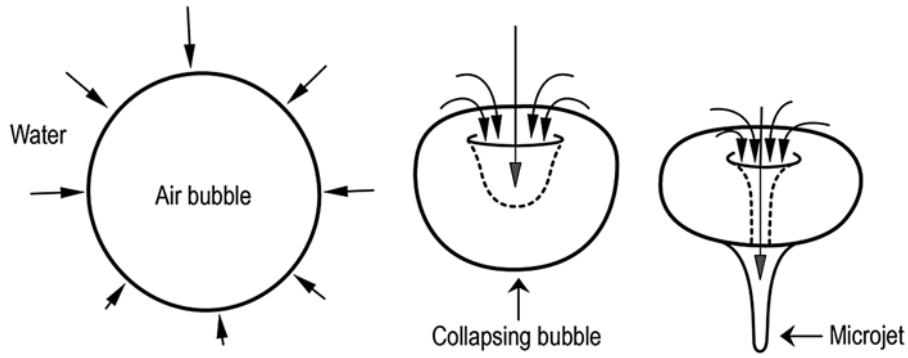

Fig. 21.2 Sketch of an inward collapsing air bubble immersed in a fluid and formation of a fluid high-speed microjet

21.3 Material and Methods

21.3.1 Shock Wave Source

A piezoelectric, experimental shock wave generator, based on a *Piezolith 2300* extracorporeal shock wave lithotripter (Richard Wolf GmbH, Knittlingen, Germany), was designed to transform filamentous fungi. The device consists of approximately 3,000 piezoceramic crystals arranged on a bowl-shaped aluminum backing, insulated from water by a flexible polymeric material (Fig. 21.3). The distance from the spherical shock wave source to its center *F* is 345 mm. Application of a high voltage discharge to the array results in the sudden and simultaneous expansion of all crystals. The pressure pulses formed by the crystals travel towards the center and generate a shock wave in the vicinity of *F*. The electric circuit consists of a 0.5 μF capacitor charging unit and a discharge control system. A high-voltage power supply charges the capacitor. It remains charged until the spark gap is fired and the stored energy is discharged towards the piezoelectric array. A spark gap-trigger switch driven by a special pulse generator is used to control the discharge frequency. The shock wave generation rate and the discharge voltage can be varied from 0.1 to 1.0 Hz and from 4.8 to 9.1 kV, respectively. A Lucite water tank (with a 675-mm × 675-mm base and a height of 450 mm) and a XYZ positioner were placed on top of the shock wave generator. Degassed water was used as coupling media to transfer the acoustic energy into polyethylene bags containing conidia suspension. A special holder to fasten and center the bags horizontally at the focus *F* was manufactured. The error in positioning was estimated to be less than 1 mm. The system was operated in repetition mode at a rate of 0.5 Hz. The water level and the water temperature were set to 80 mm above *F*, and 23 °C. The mean positive and negative pressure values, recorded with a polyvinylidene fluoride needle pressure gauge (Imotec GmbH, Würselen, Germany), having a 20 ns rise time, connected to a 300 MHz digital oscilloscope (Tektronix, Inc., Beaverton, OR, USA, model TDS3032) were 37.8 ± 4.2 MPa and 18.2 ± 2.4 MPa, respectively (mean ± standard deviation). The dynamic focus had the shape of a cigar measuring approximately 17 × 3 × 3 mm.

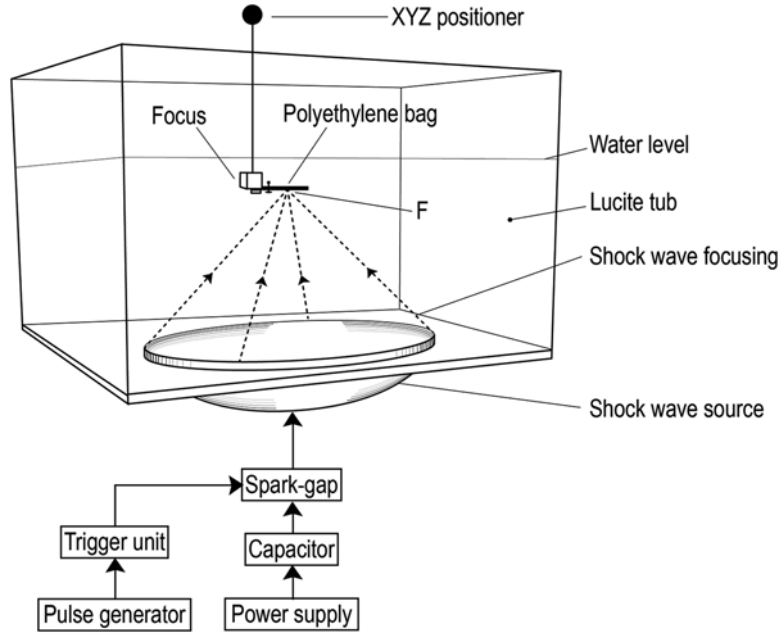

Fig. 21.3 Simplified diagram of the experimental setup employed to transform filamentous fungi

21.3.2 Transformation Protocol

As an example of shock wave-mediated transformation, a general protocol to transform intact conidia of *A. niger* is described in this section.

21.3.2.1 Preparation of Conidia of *A. niger*

1. Inoculate 1×10^3 conidia of *A. niger* in agar minimal medium; this medium contains glucose 1 %, 50 mL of nitrate solution (120 g of $NaNO_3$, 10.4 g of KCl, 10.4 g of $MgSO_4 \cdot 7H_2O$, and 30. 4 g of KH_2PO_4 per liter in distilled water), 100 μL of thiamine 1 % in distilled water, 100 μL of trace elements (2.2 g of $ZnSO_4 \cdot 7H_2O$, 1.1 g of H_3BO_3, 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.17 g of $CoCl_2 \cdot 6H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$, 0.15 g of $Na_2MoO_4 \cdot 2H_2O$, and 5 g of Na_4EDTA in 60 mL of distilled water), and 18 g of agar per liter.
2. Incubate the plates at 30 °C for 5 days.
3. Harvest the conidia with 5 mL of liquid minimal medium.
4. Vortex the conidial suspension to disaggregate the cells.
5. Incubate the plates at 30 °C for 5 days.
6. Harvest the conidia with 5 mL of liquid minimal medium for *Aspergillus*.
7. Vortex the conidial suspension to disaggregate the cells.

21.3.2.2 Preparation of the Samples

1. Adjust the concentration of conidia using a hemocytometer to a final concentration of $1–5 \times 10^3$ viable conidia per milliliter. It is important to verify the viability of the conidia when preparing the sample.
2. Elaborate 15 × 10 mm heat sealed bags using commercial polyethylene bags (Ziploc™, SC Johnson, Racine, WI, USA). Polyethylene allows adequate shock wave transfer into the conidial suspension.
3. Prepare the control sample using 200 μL of conidial suspension without recombinant DNA.
4. Add the recombinant DNA to conidial suspension to reach a final concentration of 50 μg/mL of DNA.
5. Elaborate samples with 200 μL of conidial suspension with recombinant DNA using the heat sealed plastic bags. The presence of air must be avoided in each sample, because air impairs adequate transfer of the shock wave energy to the cells.

21.3.2.3 Shock Wave Treatment

1. Fasten the bags horizontally inside the water tank of the shock wave generator; the focus *F* should be centered inside the bag (see Fig. 21.3).
2. Set the water level 80 mm above the focus *F*.
3. Expose each sample to 50, 100, 200, 300, or 400 shock waves generated at 7.5 kV at a rate of 0.5 Hz.
4. Recover the conidial suspension by transference to a clean, centrifuge tube with a sterile tip and inoculate on 3 M cellulose filters (Millipore, Plano, TX, USA) placed on minimal medium agar plates without selection.
5. Incubate the cultures at 28 °C for 24 h. This step allows the regeneration of conidia and the expression of recombinant genes in significant levels.
6. Transfer the filters to fresh minimal medium plates in the presence of selective agent. The colonies should be visible by the fifth day of incubation.
7. Propagate the colonies in agar minimal medium with a selective agent to verify the resistance of the colonies obtained.
8. Incubate at 30 °C for 5 days to recover conidia.
9. Recover the conidia (1×10^3 per mL) obtained in the previous step in liquid minimal medium (100 mL) for DNA extraction and subsequent molecular analysis.

21.4 Results and Discussion

Using the techniques described above, we have performed several experiments of genetic transformation employing conidia of *A. niger* ATCC 1015 and other filamentous fungi. This method allows the use of fresh, viable conidia without previous treatment. In addition, a much lower number of conidia are required ($1–5 \times 10^3$ per mL as opposed to the usual 1×10^6 per mL).

Example 1

Generation of *A. niger* strains resistant to hygromycin. The plasmid pANGFPHPH, which contains the *gpdA* promoter of *A. nidulans* fused to the hygromycin resistance gene (*hph*) and the *trpC* terminator of *A. nidulans* was used for transformation. A fragment of the *gpdA* promoter, the sequence of the green fluorescent protein (GFP), and the NOS terminator were joined to these sequences (Magaña-Ortiz et al. 2013). The plasmid was propagated in *Escherichia coli* DH5α and extracted according to standard procedures (Sambrook et al. 1989).

Two control groups were used. Samples in control group 1 were exposed to shock waves but did not contain recombinant DNA. Bags in control group 2 contained recombinant DNA but were not treated with shock waves. All experiments were performed in triplicate. In order to investigate the reduction of viability due to shock wave treatments, three samples with conidial suspension and recombinant DNA were grown without selection. Viability was significantly reduced at high number of shock waves regardless of the presence or absence of recombinant DNA.

Typically, transformants were visible after 5 days of incubation at 28 °C and were transferred to fresh selective media three times. After this, the colonies were propagated in liquid selective media up to twenty times to confirm the antibiotic resistance. To evaluate the stability of gene insertion, the colonies were grown in ten consecutive occasions in liquid minimal medium without hygromycin and the DNA was extracted to perform molecular analysis.

Resistant colonies were only obtained when using 100 or 200 shock waves (Table 21.1). Furthermore, in the control groups spontaneous resistance to antibiotic was not observed. The application of 200 shock waves reduced the transformation frequency by 50 %, in comparison with 100 shock waves (Table 21.1). We hypothesize that this reduction in cell viability by one order of magnitude (from 1×10^4 to 1×10^3 colonies) was caused by the increased number of shock waves, which, at high number may be damaging the fungal cells in some way and the recombinant DNA present in the samples (Campos-Guillén et al. 2012). In spite of this, the number of resistant strains was high enough for subsequent analysis.

Table 21.1 Number of hygromycin-resistant colonies obtained using a different number of shock waves

No. of shock waves	No. of hyg-resistant colonies
0[a]	0
50	0
100	19.333 ± 3.055
200	0.000 ± 2.000
300	0
400	0

A total volume of 200 μL with 1×10^4 conidia and 10 μg of recombinant plasmid were used. Results are reported as the mean ± standard deviation ($N = 3$)

[a]Control with DNA

When the transformation frequency per microgram of DNA obtained with the shock wave method was compared with that from the available protocols of genetic transformation like electroporation, the values obtained were very low. For example, Ozeki et al. (1994) obtained 100 colonies per microgram in comparison with the maximum of 2.2 colonies obtained with shock waves. However, the number of colonies generated using shock waves was 5,400 and 280 times higher than that obtained with *Agrobacterium* and protoplast transformation, respectively, when the comparison was based on the number of cells used (de Groot et al. 1998; Meyer et al. 2007). We need to include high amounts of DNA for the reason described above.

DNA extraction from fungal transformants was performed according to Punekar et al. (2003). DNA obtained of randomly hygromycin-resistant colonies was used for the first screening PCR. The oligonucleotides 5′-GCACGAGGTGCCGGA-3′ (forward) and 5′-GCTCTCGGAGGGCGA-3′ (reverse) were used to amplify a fragment of the hph gene. This fragment was detectable in the hygromycin-resistant colonies but not in the wild type strain. Southern blot analysis of nine transformants showed the insertion of one copy of hph DNA in three cases and two copies of transgenic DNA in the other six transformants (Fig. 21.4). This result showed that by using shock waves it is possible to obtain transgenic strains with single insertions and GFP activity (Fig. 21.5).

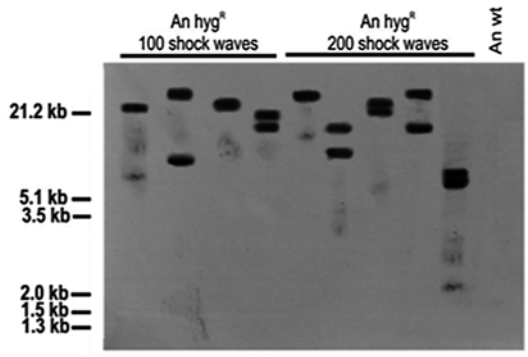

Fig. 21.4 Southern blot analysis of nine independent transformants obtained by shock wave method. A non-transformed culture of *Aspergillus niger* was used as the negative control. An α-^{32}P-dCTP-labeled fragment of the *hph* gene from pANGFPHPH was used as a probe. DNAs of these samples were digested with *EcoRI*, which cut only once in the *hph* fragment. In addition, the green fluorescent protein was observed in the transgenic strains but not in the wild type strain (An wt) (Fig. 21.5)

Example 2

Generation of *A. niger* strains with reduced proteolytic activity. *A. niger* is a versatile cell factory of numerous compounds like metabolites and enzymes. Its GRAS (Generally Recognized as Safe) status and the high levels of secretion of endogenous proteins (up to 20 g/L) make it an ideal platform for production of heterologous proteins like antigens and enzymes (Meyer 2008; Fleissner and Dersch 2010). However, the use of *A. niger* for the heterologous expression is hampered for several reasons. The main limitation is the low frequency of transformation achieved using standard methods of gene delivery. In many instances, this problem is overcome by repeating the transformation until a sufficient number of transformants is obtained, but this approach may not work for instance for generation of libraries of randomly tagged mutants. In addition, the

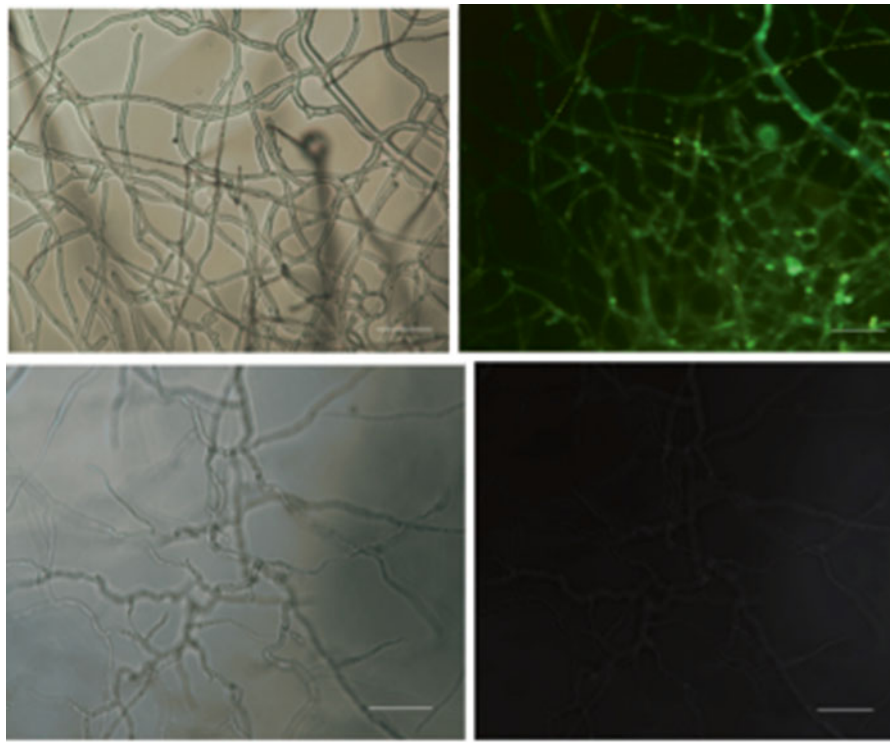

Fig. 21.5 Bright field (*left*) and fluorescence micrographs (*right*) of 3-day-old cultures of *Aspergillus niger*. The top two micrographs correspond to a strain transformed with GFP and the bottom micrographs correspond to a wild type strain (Scale bar: 10 μm)

endogenous proteolytic activity of the fungus may degrade the recombinant proteins produced. Low scale fermentation like shake flask cultures can promote the acidification of media and enhance proteolytic activity (van den Hombergh et al. 1997). The use of bioreactors may reduce somewhat the degradation of recombinant proteins, but a residual activity is observed. Strategies like the addition of inhibitors of proteolytic activity to the medium or the use of buffers that control acidification have been inefficient (Broekhuijsen et al. 1993; Ward et al. 2004).

In order to reduce the proteolytic activity of *A. niger* for heterologous protein production, mutant strains have been generated by exposure to ultraviolet light and mutants were isolated by halo screening in defined media (Mattern et al. 1992; van den Hombergh and van de Vondervoort 1995). The aspartyl protease (pepA) was determined as the major extracellular protease in *A. niger* (Mattern et al. 1992; Jarai and Buxton 1994). For this reason, the initial attempts were focused on the deletion of the respective gene. Punt et al. (2008) analyzed a mutant with low proteolytic activity and complemented the mutation using a cosmid library. This allowed the identification of the transcriptional regulator prtT, a member of the Zn2Cys6-binuclear cluster protein family, whose homologous are present in several *Aspergillus* species and other heterologous fungi. The analysis showed that this transcription regulator is involved in the complex network of induction of proteases in *A. niger*.

In order to interrupt the sequence of *prtT* by homologous recombination using shock waves, we amplified a sequence that contained two flanking regions of 1,000 bp of the gene *prtT*, upstream and downstream. Between these sequences a cassette containing the *gpdA* promoter, *ble* gene (that conferred resistance to the antibiotic phleomycin), and *trpC* terminator *trpC* was cloned. The whole sequence was employed to transform intact conidia of *A. niger*.

The protocol of transformation was similar to previously described using 100 shock waves and 5 μg/mL of phleomycin for selection. Conidia exposed to shock waves without the presence of recombinant DNA did not grow in selective media. After three rounds of selection the spores of phleomycin-resistant colonies were transferred to casein–gelatin media as described (van den Hombergh and van de Vondervoort 1995). A white halo will be produced by degradation of casein and gelatin in the strains with proteolytic activity, while no halo was visible around the obtained mutants (Fig. 21.6). The use of skim milk plates reported by other authors was ineffective in our hands to select the mutants with low proteolytic activity (Mattern et al. 1992).

Finally, the mutants were grown three times in casein–gelatin media to determine whether the phenotype was maintained. Four out of the 200 original transformants selected showed resistance to phleomycin and a conserved low proteolytic activity through the successive cultures (Fig. 21.6), suggesting a 2 % gene targeting. Initial rtPCR analysis confirmed the absence of prtT transcript on casein–gelatin. These mutants grew normally in minimal medium for *Aspergillus* and casein–gelatin media. Also, analysis of extracellular protein production showed that the secretion system of the mutant was not affected. These colonies may be useful for heterologous gene expression in *A. niger*.

21.5 Conclusions

The development of new methods and applications in biotechnology demands novel strategies to genetically transform and manipulate the fungal genome. Incorporation of specific sequences into the fungal genome in an easy, safe, reliable, and reproducible form is essential to improve their characteristics and obtain a specific phenotype. Genetic transformation of fungi currently faces major challenges. We need a better understanding of the phenomena involved in genetic transformation besides the need for new promoters and regulatory sequences both constitutive and inducible to devise more rigorous protocols and open new strategies for genetic transformation to enhance heterologous gene expression. The growing availability of genomic information should help towards this goal.

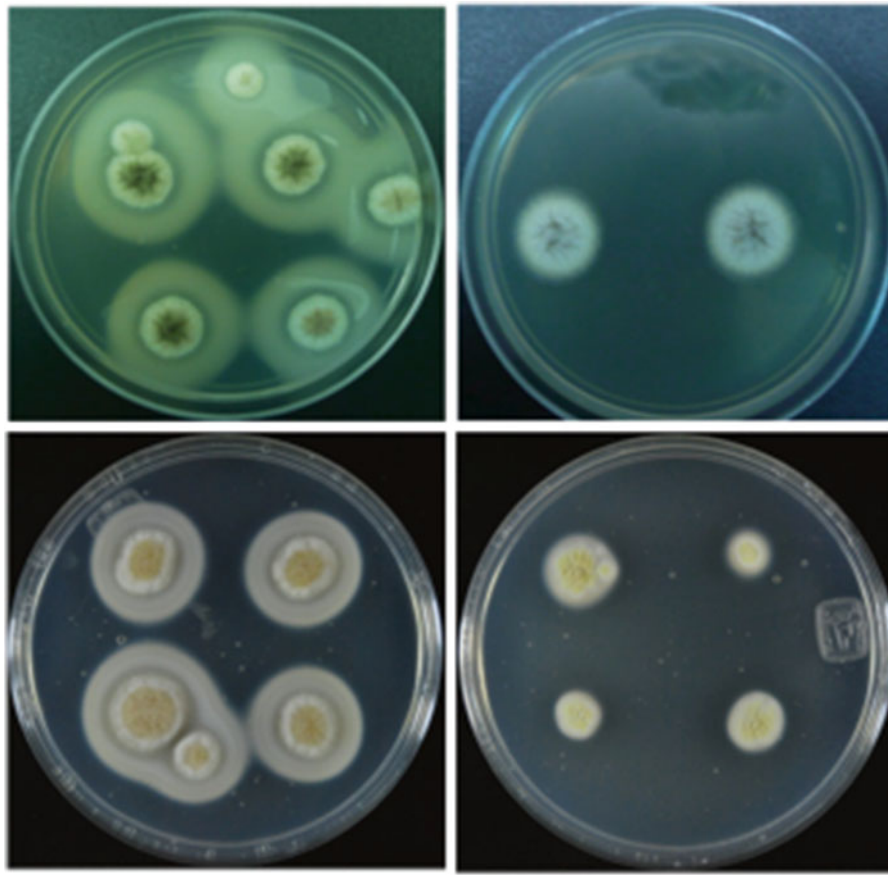

Fig. 21.6 Proteolytic activity of *Aspergillus niger* wild type and mutant strains in casein–gelatin media. The medium used for growth is composed of casein and gelatin. The white halo (*top*, *bottom left*) is caused by precipitation due to proteolytic activity. The halo is absent in the mutants (*top*, *bottom right*) indicating a reduced proteolytic activity. In contrast to wild type strains, growth of hyphae is clearly discernable in areas surrounding the mutant strains. *Left*: *A. niger* wild type strain. *Right*: *A. niger* Δ*prtT* mutant

Shock wave-mediated transformation is an attractive alternative for efficient fungal transformation, but it is necessary to develop more methods both physical and biological to increase the battery of tools for an optimal introduction of heterologous or homologous genes into fungi with high efficiency.

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Part VIII

Exogenous DNA: Uptake of DNA

Pathways and Mechanisms of Yeast Competence: A New Frontier of Yeast Genetics

22

Petar Tomev Mitrikeski

22.1 Introduction

Uptake of genetic material into a cell that results in a heritable change defines the phenomenon of genetic transformation (Griffith 1928; Avery et al. 1944). However, in order to accept exogenous DNA the cell must first be rendered competent. Contrary to many (or all?) prokaryotes, where genetic transformation is considered to be an evolutionary adaptation (Maynard Smith et al. 1991; Denamur et al. 2000; Kohiyama et al. 2003), in eukaryotes usually application of man-made technology is necessary to provoke the phenomenon. However, yeast might also become competent in laboratory conditions matching natural fungal environment(s) (Costanzo and Fox 1988; Heinemann and Sprague 1989; Nishikawa et al. 1990; Bundock et al. 1995; Piers et al. 1996; Sawasaki et al. 1996; Nevoigt et al. 2000; Hooykaas et al. 2006; Soltani et al. 2009). Such spontaneous competence has suggested that artificial eukaryotic transformation relies on naturally occurring cellular processes. Several recent investigations have started to unravel the puzzle of eukaryotic competence identifying many genes and/or entire cell processes responsible for the phenomenon (Kawai et al. 2004; Soltani et al. 2009; Riechers et al. 2009). Thus eukaryotic competence may be seen as a complex, quantitative genetic trait (Johnston et al. 1981), influenced by both the genome and its environment, which may have allowed yeast to better adopt over evolutionary times (Fitzpatrick 2012).

This chapter summarizes the pathways and mechanisms of eukaryotic competence mainly relying on knowledge acquired from yeast *Saccharomyces cerevisiae* (for further reading, see Brzobohatý and Kováč 1986; Bruschi et al. 1987; Nevoigt et al. 2000; Tomlin et al. 2000; Gietz and Woods 2001; Hayama et al. 2002; Neukamm et al. 2002; Kawai et al. 2004; Zheng et al. 2005; Hooykaas et al. 2006; Chen et al. 2008; Kawai et al. 2009; Riechers et al. 2009; Soltani et al. 2009; Kawai et al. 2010; Riechers et al. 2010; Pham et al. 2011a, 2011b; Mitrikeski 2013). Here, the intention is to define the paradigm(s), highlight the open questions and pinpoint possible new avenues for future research, while critically discussing the contemporary understandings of the phenomenon.

22.2 Pathways of Yeast Competence

Yeast cell can be made competent for exogenous DNA uptake either naturally or artificially (Table 22.1). Natural competence is either

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Table 22.1 Types of competence for exogenous DNA uptake in *S. cerevisiae*

Art/*Approach*/Method	Vehicle	Efficiency/ frequency	Reference
Artificial			
Biological			
Spheroplast-method	PEG/Ca^{2+}	~10^4	Hinnen et al. (1978), Beggs (1978), Gerbaud et al. (1979), Hsiao and Carbon (1979), Orr-Weaver et al. (1983)
Chemical			
LiAc/SS-DNA/PEG	PEG/Li^+/Heat shock	>10^6	Ito et al. (1983), Schiestl and Gietz (1989)
PEG/Cations	PEG/Li^+, Ca^{2+}, Mg^{2+}	>10^3	Keszenman-Pereyra and Hieda (1988)
PEG/Heat shock	PEG/Heat shock	>10^3	Stateva et al. (1991), Hayama et al. (2002), Chaustova et al. (2008)
PEG/freezing-thawing	PEG/Cell surface damage (?)	10^3	Klebe et al. (1983)
PEG-only	PEG	>10^2	Hayama et al. (2002)
Cations/Heat shock	Ca^{2+}/Heat shock	~10^4	Broach et al. (1979)
Cations-only	Mg^{2+}	Ambiguous	Khan and Sen (1974)
Physical			
Electroporation	Electro diffusion	>10^6	Karube et al. (1985), Delorme (1989), Manivasakam and Schiestl (1993)
Biolistic	Ballistic force	~10^{-4}	Johnston et al. (1988), Armaleo et al. (1990), Bonnefoy and Fox (2007)
Natural			
Mediated			
E. coli	Direct cell-to-cell contact	10^{-3}	Heinemann and Sprague (1989), Nishikawa et al. (1990)
A. tumefaciens	Direct cell-to-cell contact	10^{-3}	Bundock et al. (1995), Piers et al. (1996), Sawasaki et al. (1996), Hooykaas et al. (2006), Soltani et al. (2009)
Induced			
Physiological	Sugar metabolism	1–10	Nevoigt et al. (2000)
Mechanical	Physical cell wall damage	10^3	Costanzo and Fox (1988)
Ambiguous			
Natural-competence-based transfection in yeast	Shift from hypertonic to hypotonic medium/Possibly sugar metabolism	>10^{-7}	Neukamm et al. (2002)

PEG polyethylene glycol, *ss* single-stranded, *Efficiency* transformants per microgram of DNA, *Frequency* viable spheroplasts transformed or transformants per recipient cell

biologically mediated or environmentally induced. During biological mediation, yeast becomes prone to transformation through conjugation conducted either by *Escherichia coli* (Heinemann and Sprague 1989; Nishikawa et al. 1990) or *Agrobacterium tumefaciens* (Bundock et al. 1995; Piers et al. 1996; Sawasaki et al. 1996; Hooykaas et al. 2006; Soltani et al. 2009). Moreover, both mechanical (Costanzo and Fox 1988) and physiological (Nevoigt et al. 2000) mechanisms are known to enhance natural yeast competence during environmental induction in laboratory.

On the other hand, yeast can be made artificially competent by the aid of biological (Hinnen et al. 1978; Beggs 1978; Gerbaud et al. 1979; Hsiao and Carbon 1979; Orr-Weaver et al. 1983), chemical (Khan and Sen 1974; Broach et al. 1979; Ito et al. 1983; Klebe et al. 1983; Keszenman-Pereyra and Hieda 1988; Schiestl and Gietz 1989; Stateva et al. 1991; Gietz et al. 1995; Hayama et al. 2002; Chaustova et al. 2008), and physical (Karube et al. 1985; Johnston et al. 1988; Delorme 1989; Armaleo et al. 1990; Manivasakam and Schiestl 1993; Bonnefoy and Fox 2007) manipulations which either eliminate/weaken natural obstacles that block the entrance of DNA (biological and chemical approach) or simply bridge them by electrical or biolistic force (physical).

Finally, yeast can also be transformed by nonviral DNA transfer in an elaborated process that might resemble transfection (Neukamm et al. 2002). However, this could be classified neither as artificial nor as natural art of yeast transformation due to its intrinsic ambiguousness (see Mitrikeski 2013).

22.3 Mechanisms of Exogenous DNA Uptake during Yeast Transformation

In order to transform the eukaryotic cell, exogenous DNA needs not only to enter the cell (as in prokaryotes) but to reach the nucleus as well. Therefore, on its way to the nucleus the transforming DNA (tDNA) needs to pass at least four natural obstacles: (1) the cell wall, (2) the cell membrane, (3) the cell cytoplasm, and (4) the nuclear envelope. Here, I will extensively discuss the known mechanisms allowing exogenous DNA to transform yeast cell. However, before DNA internalizes into the recipient cell it first needs to establish a proper contact with its surface. In other words, free DNA in solution is not able to transform the cell without prior attachment to its surface.

22.3.1 Attaching to the Cell Surface

Hitherto experimental knowledge suggests that during transformation only DNA attached to the cell surface can pass through (Pham et al. 2011a). Therefore, tDNA must first accomplish a closer contact with the cell surface in order to subsequently transform the cell. Accordingly, Kawai et al. (2010) suggested that tDNA initially becomes attached onto the cell wall through the indispensable role of polyethylene glycol (PEG). PEG is needed not only during chemical transformation (Bruschi et al. 1987; Gietz et al. 1995; Zheng et al. 2005) but also during biological (although there the cell bears no cell wall; Brzobohatý and Kováč 1986; Zheng et al. 2005; Chen et al. 2008) and it is known to increase the transformation efficiency during physical approach (electroporation; Manivasakam and Schiestl 1993), suggesting the importance of prior attachment. Thus proper tDNA attachment to the cell surface prior to transformation is almost ubiquitous in yeast artificial competence. The exception is the biolistic approach where tDNA gets accelerated and pushed into the cell by a physical force during which prior attachment to the cell surface plays no role.

During natural mediated yeast competence tDNA is internalized by the mediator bacteria and thus no preliminary contact between DNA and the cell surface is needed (Krüger and Stingl 2011). However, the prior-to-transformation contact between tDNA and cell surface during induced natural yeast competence is more difficult to grasp. This kind of competence occurs when wild yeast is dwelling in its natural habitats. It is plausible to expect that some DNA—mainly from decomposing surrounding sister cells but in a more advanced biosphere also form other species—is present in the environment. Moreover, such DNA should somehow attach onto the surface awaiting to transform the cell if an opportunity opens a passage (occasional mechanical or physiologically induced change of the cell wall?). Possibly, such events are rare and that might be the reason for the low efficiency of natural yeast competence. Although all this is very speculative, proper DNA attachment onto the cell surface, as a prerequisite for successful transformation, is expected to occur and probably indispensable also during natural yeast competence.

Taken together, this could suggest that improving the attraction of tDNA to the cell surface via

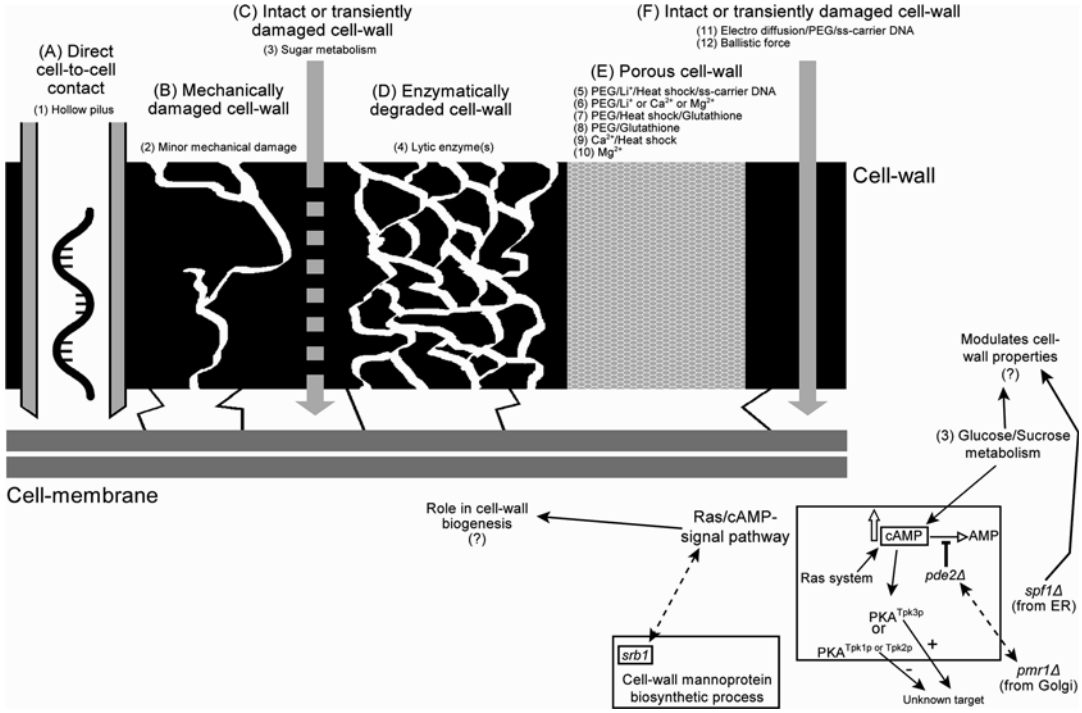

Fig. 22.1 Uptake of DNA during yeast transformation—crossing the cell wall. The Ras/cAMP pathway is expected to be involved in competence plausibly through increased level of cAMP (Kawai et al. 2004). When Tpk3p is installed on PKA competence is increased contrary to Tpk1p or Tpk2p. Sugar metabolism (monosaccharides/disaccharides) also leads to elevated level of cAMP and Ras/cAMP pathway further appears to modulate cell wall construction (*srb1*). When *SPF1* is nonfunctional yeast competence is increased probably due to enhanced DNA absorbance on the cell surface. Artificial competence: Biological: D_4, Chemical: LiAc/SS-DNA/PEG: E_5, PEG/Cations: E_6, PEG/Heat shock: E_7, PEG-only: E_8, Cations/Heat shock: E_9, Cations-only: E_{10}, Physical: Electroporation: F_{11}, Biolistic: F_{12}, Natural competence: Mediated: A_1, Physiologically induced: C_3, Mechanically induced: B_2, Natural-competence-based transfection in yeast: C_3. *ER* endoplasmic reticulum, *PEG* polyethylene glycol, *ss* single-stranded. This drawing is not in scale

more elaborated technologies might lead to better transformation efficiencies in future protocols. This is supported by the observation that intact, but not striped, cells of high-competence *spf1* mutants (Kawai et al. 2004) adsorb more tDNA on the cell surface (Pham et al. 2011a). Care should be taken that DNA is only attracted onto the surface and not fixated, since it is known that tDNA uptake appears only after PEG removal (Bruschi et al. 1987). Secondly, the well-known saturation of DNA binding sites on the cell surface will limit the maximum amount (Zheng et al. 2005).

22.3.2 Crossing the Cell Wall

It is generally believed that the cell wall is the most difficult barrier for successful yeast transformation (Fig. 22.1). We know now that both the genome and its environment (either natural or artificial) are responsible for enabling tDNA to pass it during yeast transformation. The cell wall is usually traversed via (1) direct cell-to-cell contact (Fig. 22.1A), (2) through cracks produced by physical and/or chemical damages (Fig. 22.1B, D, F), and (3) by changing its adherence-ability and/or permeability (Fig. 22.1C, E). Therefore, all transformation

strategies involve one or more of these mechanisms. During mediated natural yeast competence, tDNA usually travels a hollow pilus establishing cell-to-cell contact between the donor bacterium and the yeast cell in order to pass the cell wall (Fig. 22.1A$_1$) (Krüger and Stingl 2011).

Artificial competence will physically damage the cell wall. For example, through enzymatic digestion in order to obtain spheroplasts (Fig. 22.1D$_4$) or, alternatively, electroporation or biolistic transformation will produce transient damage (cracks) allowing tDNA to pass through (Fig. 22.1F$_{11,12}$). Under ecological conditions, weaker physical damages are expected to occur (Fig. 22.1B$_2$). We now know that the successful alteration of both chemical and physical properties of the cell wall is dependent on the action of certain genes (see *Genes responsible for yeast competence*; Gallego et al. 1993; Durand et al. 1993; Tomlin et al. 2000; Kawai et al. 2009; Pham et al. 2011a) and environmental factors (PEG, cations, heat shock, and carrier DNA) expected or known to alter cell wall properties (Brzobohatý and Kováč 1986; Bruschi et al. 1987; Zheng et al. 2005; Chen et al. 2008; Pham et al. 2011b).

Although it is plausible that both heat shock and carrier DNA are altering cell wall properties, yeast competence does not occur if either of both is applied in isolation. In contrast, PEG usually leads to successful yeast competence (Klebe et al. 1983; Hayama et al. 2002; Fig. 22.1E$_8$) but is highly dependent on the growth phase (Hayama et al. 2002). This further emphasizes the role of cell wall alteration since different life-cycle phases may impose different cell wall properties that (perhaps) need to be treated differently in order to mitigate the artificial entrance of tDNA. Passage of tDNA through the cell wall is easier by simultaneous use of PEG/cations due to improvement of its absorption-ability and/or permeability (Zheng et al. 2005; Chen et al. 2008; Pham et al. 2011b). A recent study has offered insight into such synergetic mechanism(s) by visualizing a more porous cell wall during yeast transformation after treatment with single-stranded (ss)-carrier DNA and Li^+ (Pham et al. 2011b). Transmission electron microscopy imaging suggested that carrier DNA is able to cause a structural change of the cell wall by partially entering it and the process may be synergistically enhanced by Li^+. This combines the adsorption role of PEG and the cell wall-penetrating role of ss-carrier enhanced by Li^+ (and possibly by heat shock) (Fig. 22.1E$_{5,6,7}$). It is also interesting that cations alone are known to provoke transformation during miscellaneous natural competence (Khan and Sen 1974; Broach et al. 1979; Fig. 22.1E$_{9,10}$). Although the way by which DNA gets absorbed on the cell surface here is not known, it is plausible to suggest that, once adhered, tDNA may pass the cell wall if properly altered. An example of altered cell wall during spontaneous transformation is physiologically induced natural competence in yeast. This may be the result of elevated cAMP levels due to mono/disaccharides metabolism (Tomlin et al. 2000; Kawai et al. 2004; Vandamme et al. 2012) (Fig. 22.1C$_3$). Moreover, the Ras/cAMP-signalling pathway might support competence through altered cell wall properties also during the so-called yeast transfection (Neukamm et al. 2002; Fig. 22.1C$_3$) or perhaps in overall yeast transformation. The hypo- to hypertonic shift imposed during this transfection might also affect the cell wall properties possibly leading to better DNA adherence and/or passage.

Taken together, this implies that cell wall alteration is very important for both natural and artificial yeast competence. In a broader sense, the physical damage of the cell wall—major or minor—is also an alteration. Obviously, exceptions are mediated natural competence (pilus mediated) and biolistic (physical artificial competence). There, the tDNA is either injected through a pilus or simply bombarded, respectively. However, while no preparative cell wall structural change(s) facilitating transformation is used during biolistic, the penetration of the pilus may still depend on a yet-unknown alteration of the wall. Moreover, histon deacetylation during mediated natural competence also influences yeast transformability (Soltani et al. 2009). Perhaps, some of the thereby regulated genes are involved in cell wall alteration leading to competence. Therefore, future studies of the role of cell wall alterations during yeast competence should focus

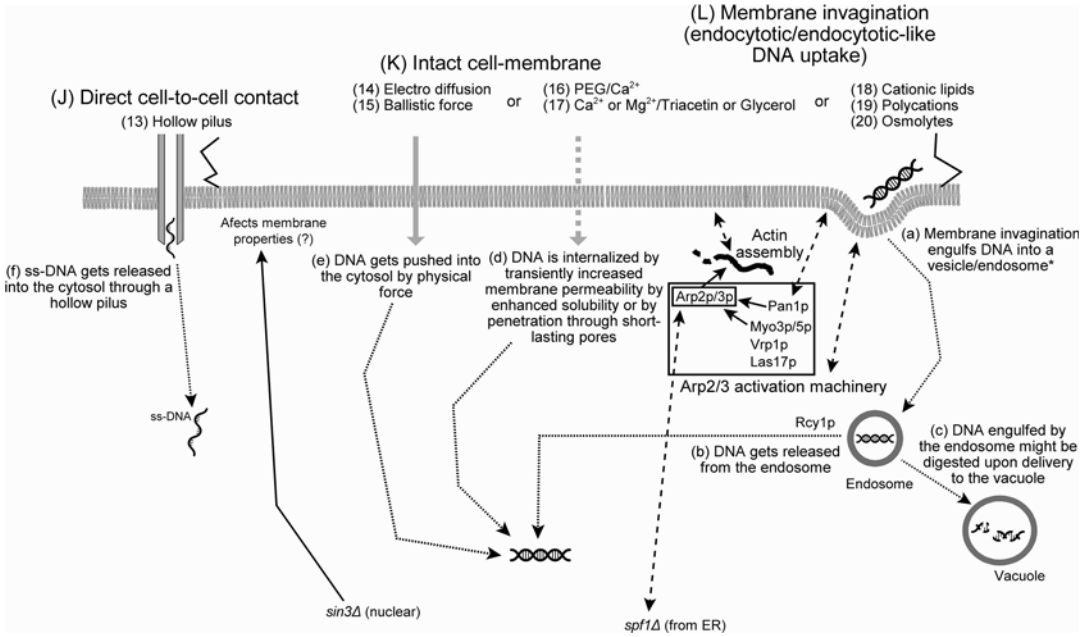

Fig. 22.2 Uptake of DNA during yeast transformation—entering the cell/surviving into the cytosol. The Arp2p/3p activation machinery might be critical for DNA internalization (Kawai et al. 2004). When *SIN3* is deleted yeast competence is decreased, probably due to lack of PE in the membrane. Endocytosed DNA is either released intact from the endosome or digested upon delivery to the vacuole. Genes like *RCY1* are involved in DNA release during early endocytotic steps (Riechers et al. 2009). Also, acidification of endosomal/vacuolar compartments differing from wild type slows down the transport of endocytosed DNA to the vacuole. Artificial competence: Biological: $K_{16}d/L_{16}ab$, Chemical: LiAc/SS-DNA/PEG: *Kd/Lab*, PEG/Cations: $K_{17}d/L_{17}ab$, PEG/Heat shock: *Kd/Lab*, PEG-only: *Kd/Lab*, Cations/Heat shock: *Kd/Lab*, Cations-only: *Kd/Lab*, Physical: Electroporation: $K_{14}e$, Biolistic: $K_{15}e$; Natural competence: Mediated: $J_{13}f$, Physiologically induced: *Kd/Lab*, Mechanically induced: *Kd/Lab*; Natural-competence-based transfection in yeast: $L_{18,19,20}ab$ (the slash in the sequences means *or*). *Note that during biological approach engulfed DNA can also be ss. *ER* endoplasmic reticulum, *PEG* polyethylene glycol, *ss* single-stranded. This drawing is not in scale

more on genomic encoded effectors (Brzobohatý and Kováč 1986; Bruschi et al. 1987; Zheng et al. 2005; Chen et al. 2008; Pham et al. 2011b).

22.3.3 Entering the Cell

tDNA that has successfully passed the cell wall now enters the space between the wall and the membrane which is the next barrier to successful transformation. However, DNA is not membrane soluble and therefore cannot enter the cell without the aid of a vehicle. Additionally, its negative charge repels it from the membrane. Few mechanisms allowing tDNA to cross the membrane and internalizes into the cell are described (Fig. 22.2). In the majority of transformation methods, tDNA traverses the membrane either by endocytosis (or an endocytosis-like process; Fig. 22.2L) or transient increased membrane permeability (Fig. 22.2K). Such permeability might be supported both by enhanced solubility (Fig. $22.2K_{16,17}$) and short-lasting pores (Fig. $22.2K_{14,15}$). Electroporation (physical artificial approach) will be dependent on short-lasting pores (Fig. $22.2K_{14}$), since electro-diffusion imaginably drives tDNA through them. Earlier it was suggested that electrically driven transfer across the membrane is a sequence of interactive, electro-diffusive and passive diffusion events (Neumann et al. 1996), labeling the process as purely artificial and not based on active

membrane engulfment. Still, there is no conclusive evidence that endocytosis or an endocytosis-like process is not involved in tDNA internalization during electroporation. For example, the electric force might just fix the DNA to the membrane surface which is then internalized by active engulfment. Contrary to this, the other physical artificial competence approach (biolistic; Fig. 22.2K_{15}) simply shoots tDNA in and no preexisting pores are necessary. The rough penetration will create temporarily a sort of pores. Furthermore, mediated natural transformation—where tDNA is transmitted through the pilus (Fig. 22.2J_{13})—also seems completely independent of endocytosis and transiently increased permeability.

So both the composition and the environment strongly influence the process of membrane passing during yeast transformation. While the transiently increased membrane permeability mainly depends on environmental changes (provoked either by human technology or a natural phenomena), endocytosis requires active cell processes encoded by the genome. Actin cytoskeleton organization, endocytotic transport, cytokinesis, membrane growth and polarity are all involved (Neukamm et al. 2002; Kawai et al. 2004; Riechers et al. 2009). It is usually believed that the cell engulfs foreign matter through actin-dependent membrane invagination, slowly resulting in a vesicle that is eventually internalized together with its content (Robertson et al. 2009). If this matter is DNA, the cell could become transformed. This process is expected to be responsible for most tDNA internalization events during yeast transfection (Neukamm et al. 2002). Although at first glance not obvious, similar internalization processes were suggested by Kawai et al. (2004, 2010) to be involved in PEG-derived competence. *First*, a relevant subset of endocytotic mutants have reduced competence (Kawai et al. 2004); *second*, increasing evidence supports endocytotic internalization of cationic lipid- or polymer-DNA complexes into mammalian cells (Elouahabi and Ruysschaert 2005; Khalil et al. 2006); and *third* (indirect evidence), negatively charged nanogold particles locate intracellularly along with membrane structures (Pham et al. 2011a). On the other hand, it is unclear why other subsets of relevant endocytotic mutants have no effect on competence when PEG is used (Riechers et al. 2009; Kawai et al. 2010). This suggests that during yeast transformation the membrane could be passed by other routes not dependent on endocytotic transport. Possibly, environmental factors can help tDNA internalization both by facilitating the adherence onto the membrane by eliminating the repulse between DNA and membrane, as well as increasing the probability of internalization by increasing DNA solubility in the membrane. For instance, PEG is known to stimulate DNA adherence also to the surface of stripped cells (Chen et al. 2008) (Fig. 22.2K_{16}, L_{16}). However, only divalent cations (but not monovalent; Chen et al. 2008) are expected to have role in overcoming the membrane barrier during tDNA internalization (Khan and Sen 1974; Hinnen et al. 1978; Beggs 1978; Broach et al. 1979; Gerbaud et al. 1979; Hsiao and Carbon 1979; Struhl et al. 1979; Keszenman-Pereyra and Hieda 1988) (Fig. 22.2$K_{16,17}$, $L_{16,17}$). Furthermore, lipid-soluble molecules—triacetin/glycerol (Keszenman-Pereyra and Hieda 1988; Fig. 22.2K_{17}, L_{17}) and cationic lipids (Wattiaux et al. 2000; Fig. 22.2L_{18})—are known to enhance tDNA internalization. Cationic compounds (lipid-soluble polycations; Wattiaux et al. 2000) (Fig. 22.2L_{19}) can also increase competence but it is unknown whether this is a consequence of increased solubility or neutralized charge or both. Yeast membrane composition is known to affect competence, as mutants lacking phosphatidylethanolamine (PE) (Kawai et al. 2004) due to a nonfunctional *SIN3* gene (Elkhaimi et al. 2000) show a reduced competence. Disturbances in the lipid composition of the membrane are also shown in other competence-affecting mutants (*srb1-1*; Stateva et al. 1991).

So, apart from mediated natural and physical artificial competence, transformation strategies rely mainly on two routes for passing the barrier in order to successfully internalize tDNA. However, it is still unclear whether there both processes (the endocytotic and the passive) or only one membrane-crossing route (the endocytotic) is most critical in eukaryotic transformation.

22.3.4 Surviving the Cytosol

The cytosol is a very hostile environment for free DNA. Internalized tDNA must escape destruction before entering the nucleus in order to transform the cell. Depending on the transformation strategy, internalized tDNA dwells the cytosol either in free form or protected in a membrane sac. During mediated natural and physical artificial transformation approaches tDNA is expected to exist in free form (Fig. 22.2e, f), while in most other approaches packed tDNA is more plausible (Fig. 22.2a). Nevertheless, free tDNA is also expected if internalization is facilitated by passive membrane penetration (Fig. 22.2d) rather than by engulfment.

The destiny of the internalized tDNA is believed to be influenced by gene products involved in modifying endocytotic/vacuolar pH-conditions, autophagy, and ALP sorting pathway (Riechers et al. 2009). It was proposed that non-wild type (wt) acidification of the endosome slows down the transport of tDNA, simultaneously enhancing its accumulation and delaying its delivery to the vacuole where degradation takes place (Riechers et al. 2009; Fig. 22.2c). Environmental factors can have the same effect since lysosomotropic compounds such as chloroquine are known to inhibit the transfer from endosomes to vacuole (Mellman et al. 1986). Furthermore, endocytosed tDNA needs to be released from the endosome in order to reach the nucleus (Fig. 22.2b) which was reduced in a targeted low-competence mutant (Fig. 22.2b; Riechers et al. 2009). Efficient tDNA liberation from the endosome can be induced by hypotonic shift causing swelling and rupture of the vesicles due to water influx (Neukamm et al. 2002; Riechers et al. 2009).

Significant pH shifts were reported as competence promoting factor during miscellaneous natural transformation (Hayama et al. 2002). Similarly, altered pH reduces the transport of hydrolases into the endosome/vacuole which additionally protects the packed DNA from degradation (Neukamm et al. 2002; Riechers et al. 2009); several gene products are responsible for this process (Riechers et al. 2009).

Altogether, our knowledge on the destiny of internalized tDNA is still fragmented and inconclusive. Future research needs to answer two questions: *first*, which mechanism (if any?) allows free tDNA to survive the lytic power of the cell, and *second*, how to prevent packed tDNA to be targeted to the vacuole, and moreover how and when it is released in the nucleus?

22.3.5 Reaching the Final Destination: the Cell Nucleus

The nuclear envelope is the final barrier that tDNA needs to overcome prior to successful transformation. Unfortunately, although very important this final step in order to achieve a heritable genetic alteration is the least understood. One possibility might be that vesicle packed tDNA is delivered to the nucleus through membrane fusion (Kawai et al. 2010; Fig. 22.3f). Alternatively, free cytosolic tDNA might utilize nuclear localization sequences (NLSs) during mediated natural competence to prevent degradation (Figs. 22.2f and 22.3a; see Lacroix et al. 2006). However, this is only expected for tDNA transfer conducted by *A. tumefaciens* and not by *E. coli* due to its prokaryotic origin. Physical induced competence also uses unprotected free tDNA (Figs. 22.2e and 22.3b). Here, tDNA is most probably physically forced direct into the nucleus either electrically (Fig. 22.3b1) or biolistically (Fig. 22.3 b2), escaping all intermediate degradation. In all transformation approaches utilizing the supposed passive internalization route (Figs. 22.2d and 22.3b), free tDNA is expected to pass the membrane and reach the nucleus as such (Fig. 22.3d). It is tempting to speculate that during some of these processes there are so many internalized molecules that irrespective of all barriers and degradation few eventually reach the nucleus (theoretically, one is sufficient).

Both the unprotected free cytosolic tDNA and the tDNA released from the vesicles need to be internalized in the nucleus. Hitherto knowledge favors two routes of nuclear internalization.

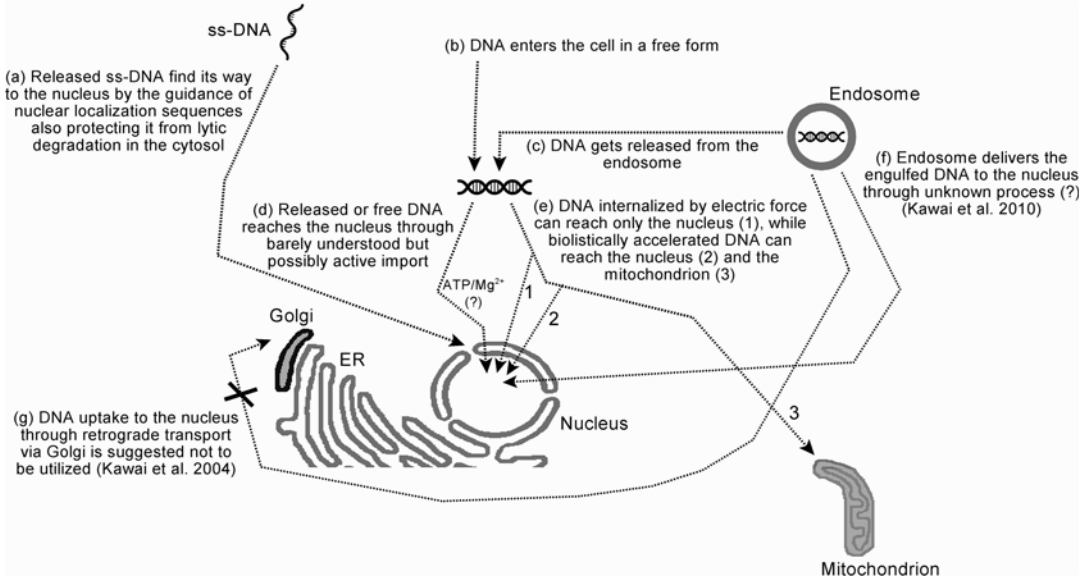

Fig. 22.3 Uptake of DNA during yeast transformation—reaching the nucleus. Artificial competence: Biological: *bd/cd/f*, Chemical: LiAc/SS-DNA/PEG: *bd/cd/f*, PEG/Cations: *bd/cd/f*, PEG/Heat shock: *bd/cd/f*, PEG-only: *bd/cd/f*, Cations/Heat shock: *bd/cd/f*, Cations-only: *bd/cd/f*, Physical: Electroporation: *be1*, Biolistic: *be2/3*; Natural competence: Mediated: *a*, Physiologically induced: *bd/cd/f*, Mechanically induced: *bd/cd/f*; Natural-competence-based transfection in yeast: *cd/f* (the slash in the sequences means *or*). *ER* endoplasmic reticulum, *ss* single-stranded. This drawing is not in scale

First, tDNA enters the yeast nucleus during mitosis when the nuclear envelope—although not broken down as in higher eukaryotes—has a more loosened structure and thus is easy to penetrate (Jaspersen and Ghosh 2012). This is supported by the fact that the highest competence is seen during S-phase (Chaustova et al. 2008), when most envelope changes are expected (Jaspersen and Ghosh 2012), and also exponentially growing cells are more efficiently transformed by nonphysically induced competence. *Second*, tDNA enters the nucleus by active import (Fig. 22.3d). It is long known that isolated yeast nuclei have the ability to uptake exogenous DNA in vitro in the presence of ATP and Mg^{2+} (Tsuchiya et al. 1988). Retrograde transport via Golgi as a way of nuclear internalization of tDNA was rejected by Kawai et al. (2004) (Fig. 22.3g).

Is the process of tDNA internalization into the cell mechanistically connected with the process of its nuclear internalization? The intrinsic coupling between these two stages is plausible during mediated natural transformation; especially with transfer mediated through *Agrobacterium*. Another imaginably predictable bond of such kind might be hidden in the overall process that is behind endosomical internalization. However, the actual mechanisms are unknown and therefore resolving the complexity of the latter phases of yeast competence is needed to further unravel the puzzle of eukaryotic transformation.

22.4 Genes Responsible for Yeast Competence

Yeast competence can be affected both by genetic and nongenetic parameters (for extensive discussion, see Mitrikeski 2013). Two main cell traits/processes are involved in yeast competence for tDNA uptake: (1) cell surface characteristics, and (2) internalization and transport (Table 22.2). The rest is difficult to articulate and classified as (3) miscellaneous.

Table 22.2 Genes involved in exogenous DNA uptake in *S. cerevisiae*

Cell trait or process/ *Sub-process*/Gene (ORF)	Gene function	Competence	Reference
Cell surface (cell wall and/or cell membrane) characteristics			
acr mutants[a]	Resistant to aculeacin A, which inhibits ß-glucan synthesis	Increased	Gallego et al. (1993)
SPF1	ER function and Ca^{2+} homeostasis	Increased	Pham et al. (2011a)
PDE2	cAMP level	Increased	Tomlin et al. (2000), Kawai et al. (2004)
SRB1	Synthesizes GDP-mannose in cell wall biosynthesis/Required for normal cell wall structure	Increased	Tomlin et al. (2000)
Unknown *SRB* gene/ allele	Synthesizes GDP-mannose in cell wall biosynthesis/Required for normal cell wall structure	Increased	Tomlin et al. (2000)
SIN3	Histone deacetylation	Decreased	Kawai et al. (2004)
Bulk material internalization and transport			
Cytoskeletal dynamics and/or organization and endocytosis Vesicular transport			
GCN5, *NGG1*, *YAF9*, *EAF7*	Involved in histone acetyltransferase-complexes	Increased	Soltani et al. (2009)
HST4, *HDA2*, *HDA3*	Involved in histone deacetylase-complexes	Decreased	Soltani et al. (2009)
GCN5, *NGG1*, *EAF7*	Involved in histone acetyltransferase-complexes	Increased[b]/ Decreased[c]	Soltani et al. (2009)
YAF9	Involved in histone acetyltransferase-complexes	Decreased	Soltani et al. (2009)
HDA3	Involved in histone deacetylase-complexes	Increased	Soltani et al. (2009)
SHE4	Regulation of myosin function	Decreased	Kawai et al. (2004)
ARC18	Actin motility and integrity	Decreased	Kawai et al. (2004)
PDE2	cAMP level	Increased	Kawai et al. (2004)
SPF1, *PMR1*	Ca^{2+} or Ca^{2+}/Mn^{2+} metabolism	Increased	Kawai et al. (2004)
VPS21, *VPS45*, *VAM6*	Endocytic transport	Increased	Riechers et al. (2009)
YPT7, *YPT51*	Endocytic transport	Increased	Neukamm et al. (2002)
STV1, *VPH1*, *NHX1*	Controlling endocytic/vacuolar pH-conditions	Increased	Riechers et al. (2009)
VPS17, *APL5*, *VAC8*, *PEP4*	Roles in autophagy and ALP sorting pathway	Increased	Riechers et al. (2009)
Miscellaneous			
GSH1	Glutathione biosynthesis	Decreased	Hayama et al. (2002)
MAT (Heterozygote; *a*/*α*)	Mating type locus	Increased	Durand et al. (1993)
Other[d]			
LAP4, *TPO2*, YGR071C, YNR061C, YDR119W	Vacuolar gene		Kawai et al. (2009)
NCE103	Protein export pathway		Kawai et al. (2009)
ATG8	Autophagy		Kawai et al. (2009)
ATX2	Mn^{2+} homeostasis		Kawai et al. (2009)
SBE2, *AXL2*, *MUB1*, YBR267W	Bud-forming		Kawai et al. (2009)
GIS4	cAMP-signal pathway		Kawai et al. (2009)

(continued)

Table 22.2 (continued)

Cell trait or process/ *Sub-process*/Gene (ORF)	Gene function	Competence	Reference
YER113C	Golgi-ER transport vesicles		Kawai et al. (2009)
NSR1	NLS binding protein		Kawai et al. (2009)

[a]Possible *ACR* gene has not been physically mapped in the sequence of yeast strain S288C (see http://www.yeastgenome.org/)
[b]As linear DNA fragment
[c]As YCp
[d]These genes are potentially important for yeast competence based on their PEG-related genome wide expression profile (see text)
ER endoplasmic reticulum, *PEG* polyethylene glycol

The main factor influencing cell competence is obviously the characteristic(s) of its surface (cell wall and/or cell membrane). Several gene products affecting transformation are connected to this (Table 22.2). They are involved in regulating the function of endoplasmic reticulum (ER) and Ca^{2+} homeostasis (*SPF1*; Pham et al. 2011a), maintaining of cAMP level (*PDE2*; Tomlin et al. 2000; Kawai et al. 2004), and the synthesis of GDP-mannose during cell wall biosynthesis (*SRB1*; Tomlin et al. 2000) or ß-glucan synthesis (*acr* mutants; Gallego et al. 1993). *SIN3* is possibly regulating many genes through histone deacetylation. Some of downstream regulated genes may be relevant for (re)shaping the cell surface during transformation based on its role in the mating-type switch (Sin3-Rpd3 histone deacetylase-complex; Wang et al. 1990) (Fig. 22.2). However, Sin3p might also affect yeast competence by shaping membrane properties through regulation of the PE content (Elkhaimi et al. 2000; Kawai et al. 2004). Furthermore, spontaneous tDNA uptake is known to be dependent on sugar metabolism (Nevoigt et al. 2000; Neukamm et al. 2002) where cAMP level seems important by altering the cell wall properties (Tomlin et al. 2000; Kawai et al. 2004; Vandamme et al. 2012). Deleting *PDE2* and *SRB* affecting the RAS/cAMP signal also corroborates its role in cell wall biogenesis (Tomlin et al. 2000) (Fig. 22.1).

Internalization and transport are the next steps important for yeast competence (Table 22.2). Many genes known to be responsible for yeast competence are involved in cytoskeletal dynamics and/or organization and endocytosis (*GCN5*, *NGG1*, *YAF9*, *EAF7*, *HST4*, *HDA2*, *HDA3*, *SHE4*, *ARC18*, *PDE2*, *SPF1*, *PMR1*; Kawai et al. 2004; Soltani et al. 2009) and others are contributing to vesicular transport (*VPS17*, *VPS21*, *VPS45*, *VAM6*, *YPT7*, *YPT51*, *STV1*, *VPH1*, *NHX1*, *APL5*, *VAC8*, *PEP4*; Neukamm et al. 2002; Riechers et al. 2009). Some low-competence genes from the first group (*SHE4*, *ARC18*; Kawai et al. 2004) and all genes from the second group (high-competence) were suggested to be involved in the endocytotic pathway of transformation emphasizing the importance of this route (see Table 22.2). Both low-competence genes are involved in the upstream phases (DNA adsorption and/or internalization), while the high-competence genes seem more important for the downstream phases (successful cytosolic DNA survival and/or endosomic release) of the endocytotic pathway.

Several of the high-competence genes from the first group are involved either in controlling the cAMP level (*PDE2*; Kawai et al. 2004; see Fig. 22.1) or in Ca^{2+} or Ca^{2+}/Mn^{2+} metabolism (*SPF1*, *PMR1*; Kawai et al. 2004). Why these processes are important for yeast competence is not entirely clear. Could the divalent cation metabolism also be involved in overcoming the cell surface as barrier during transformation? More tDNA was shown to be absorbed on the cell surface of *spf1* mutants (Pham et al. 2011a) and this gene is known to be involved in Ca^{2+} homeostasis. Moreover, only divalent cations are known to have role in traversing the membrane during transformation (Khan and Sen 1974;

Hinnen et al. 1978; Beggs 1978; Broach et al. 1979; Gerbaud et al. 1979; Hsiao and Carbon 1979; Struhl et al. 1979; Keszenman-Pereyra and Hieda 1988) (Fig. 22.2$K_{16,17}$, $L_{16,17}$).

Histone acetylation/deacetylation through genes like *GCN5*, *NGG1*, *YAF9*, *EAF7*, *HST4*, *HDA2*, *HDA3* affects mediated natural transformation by *Agrobacterium* suggesting highly specific role(s) (Soltani et al. 2009; see Table 22.2). However, histone regulation may also play an important role in other types of yeast competence since majority of these genes also impact PEG-dependent artificial transformation (LiAc/SS-DNA/PEG; *GCN5*, *NGG1*, *YAF9*, *EAF7*, *HDA3*; Soltani et al. 2009).

Finally, there are reports of genetic contribution to yeast competence but not easily attributable to a known cell trait and/or process. Significantly compromised competence was seen in glutathione-deficient cells (*GSH1* gene; Hayama et al. 2002), while strains heterozygous for the mating type locus (*MAT a/α* genotype; Durand et al. 1993) showed elevated transformability. Furthermore, genome wide expression studies of cells grown either with or without PEG revealed many genes potentially involved in competence (Kawai et al. 2009). Among the up-regulated genes were a vacuolar gene (*LAP4*), genes involved in protein export (*NCE103*) and autophagy (*ATG8*). Interestingly, numerous down-regulated genes were identified in various classes of cell metabolism: Mn^{2+} homeostasis (*ATX2*; see Kawai et al. 2004), bud-forming process (*SBE2*, *AXL2*, *MUB1*, YBR267W; due to their possible role in cell surface shaping), cAMP-signal pathway (*GIS4*; see Kawai et al. 2004), and Golgi-ER transport vesicles (YER113C; due to its role in cellular adhesion). Particular interesting leads are four vacuolar genes (YGR071C, YNR061C, YDR119W, and *TPO2* due to its role in polyamine transport since polyamines are known to stimulate plasmid DNA uptake; see Ito et al. 1983) and one nuclear gene (*NSR1*, encoding an NLS binding protein possibly important for tDNA nuclear internalization). The actual function of these genes in yeast competence needs to be elucidated.

22.5 Conclusions

Yeast transformation is one of the cornerstones of eukaryotic genetics. This powerful technology has allowed unprecedented genome manipulations, important for both fundamental and applied research. However, we still are addressing questions about its fundamentals. Luckily many undertook various efforts to acquire basic knowledge (Nevoigt et al. 2000; Kawai et al. 2004; Riechers et al. 2009; Soltani et al. 2009). This taught us that artificial eukaryotic transformation depends mainly on naturally occurring cellular processes. Alongside, this elucidated comprehensive mechanisms of natural competence involved in spontaneous yeast transformation under environmental conditions. Accordingly, these mechanisms may have allowed the yeast *S. cerevisiae* to adopt better over evolutionary times (Fitzpatrick 2012; Mitrikeski 2013). On the other hand, the insights have enabled us to artificially perfect the process such that contemporary yeast transformation is a child's game in our current laboratory practice (Gietz and Schiestl 2007a, 2007b, 2007c, 2007d, 2007e). However, this is hardly the case for many non-*Saccharomyces* species although significantly important to humanity (see Kawai et al. 2010). Therefore, knowledge on eukaryotic competence is still only partial, inconclusive and not comprehensive. For instance, we can contemplate certain cell traits and/or processes being important for eukaryotic transformation and even pinpoint certain genes (see Table 22.2). And although we can line up all transformation steps—from the initial DNA adsorption on the cell surface to the final nuclear internalization producing a heritable change—we still lack fundamental knowledge to logically assemble them. Thus the full process is expected to be concerted, as the high transformation efficiency established in some artificial approaches could not have come only from our technological advancements, as it must be connected to intrinsic elements of competence. Obviously, many important questions still remain open rendering the subject of eukaryotic competence for exogenous DNA uptake open to a more systematic and targeted approach.

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23 Evaluation of Competence Phenomenon of Yeast *Saccharomyces cerevisiae* by Lipophilic Cations Accumulation and FT-IR Spectroscopy. Relation of Competence to Cell Cycle

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23.1 Introduction

Direct insertion and integration of functionally active genes into eukaryotic cells is now a commonplace procedure which has a wide application in molecular biology and genetics. The efficiency of the process depends on the physiological state of recipient cells which is named competence. Competence is a complex of properties affected by the physiological treatment and genetic events (Ito et al. 1983; Gietz et al. 1992; Hayama et al. 2002; Mitrikeski 2013). Generally, competence is a subject of regulatory modalities such growth stage specific, nutritional responsive, and cell type specific.

Saccharomyces cerevisiae does not naturally takes-up DNA from its environment but can be made competent by chemical and enzymatic treatment, or by pulsed electrical field (Brzobohaty and Kovac 1986; Eynard et al. 1997; Gietz et al. 1992; Ito et al. 1983; Meilhoc et al. 1990; Suga et al. 2001). Yeast transformation includes the attachment of exogenous DNA onto the cell wall followed by penetration into the cell. Factors facilitating DNA binding and penetration through the cell wall, evoke significant increase of transformation efficiency. We assign the competence state of yeast cells as a capability of cells to take up exogenous DNA. It is expressed as transformation efficiency (the number of transformants per microgram of plasmid DNA).

Induction of competence in yeast cells after treatment with Li cations was studied by lipophilic cations accumulation and by FT-IR spectroscopy (Rotenberg 1997; Naumann 1998). The changes in cell wall structure induced by Li^+ ions during the cell cycle were investigated. The presence

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of natural competence in yeast was also observed (Chaustova and Zimkus 2004).

In this chapter we are summarizing data from our previous publications related to the evaluation of factors which predetermine the competence state of yeast *Saccharomyces cerevisiae*, peculiarities of yeast cell wall structure and impact of Li^+ cations in development of competence.

23.2 Permeability Properties and Transformation Efficiency of *Saccharomyces cerevisiae* Strains with Defects in Cell Wall Structure

Lipophilic cations, such tetraphenylphosphonium (TPP^+) or fluorescent lipophilic dyes are frequently used as probes for the estimation of membrane potential ($\Delta\psi$) of prokaryotic and eukaryotic cells, organelles, and vesicles. The use of lipophilic cations for the determination of membrane voltage in intact plant and fungal cells was causing some controversy, as in cell-walled species the equilibrium and steady-state distribution of lipophilic cations are complicated, and only indirectly image $\Delta\psi$ (Boxman et al. 1982; Gásková et al. 1998; Rotenberg 1997). The differences in uptake rate of these lipophilic ions reflect a number of morphological dissimilarities in structure and/or thickness of cell wall (de Nobel et al. 1990). The measurement of membrane potential using the equilibration of lipophilic cations or fluorescent probes between the cell and the external medium, is often hampered by the barrier properties of the cell wall (Ballarin-Denti et al. 1994).

An electrode selective to TPP^+ ions was applied to evaluate permeability properties of various *S. cerevisiae* strains with intact and defective wall structure:

- SEY6210: parental yeast strain with intact cell wall structure was used as parental one (Roemer and Bussey 1991).
- SEY6210 (*kre1*): yeast strain with mutation in *KRE1*, encoding secreted protein involved in β-1,6-D-glucan assembly (Boone et al. 1990). *KRE1* gene is a serine/threonine-rich secretory pathway protein with a C-terminal hydrophobic tail. Mutants of *KRE1* gene make reduced levels of β-1,6-D-glucan, which is smaller and has an altered structure as compared to wild-type (Boone et al. 1990; Brown et al. 1993; Lu et al. 1995).
- SEY6210 (*kre2*): yeast strain with mutation in *KRE2*, a mannosyl transferase required for correct O-linked glycosylation of mannoproteins (Ballou 1990; Hausler et al. 1992; Hill et al. 1992). Mutants with defects in *KRE2* grew quite well (Roemer et al. 1994).
- SEY6210 (*kre6*): the *kre6* mutation reduces the levels of both β-1,3-D-glucan and β-1,6-d-glucan in the cell wall, but does not affect the size of β-1,6-D-glucan (Roemer and Bussey 1991; Lu et al. 1995). *KRE6* encodes a predicted type II membrane protein required for glucan synthesis in vivo and for glucan synthase activity in vitro. Disruption of *KRE6* results in slow growth and to 50 % reduction of β-1,6-D-glucan present in cell wall of yeasts (Roemer and Bussey 1991; Roemer et al. 1994).
- XCY42-30D (*mnn1*): strain with a defective synthesis of α-1,2-mannosyl transferase, which is involved in protein *O*-glycosylation and is causing some structural changes in the mannan–protein complex (Raschke et al. 1973).
- LB3003-JAa (*mnn9*): strain possessing a *mnn9* mutation, unable to elaborate *N*-linked core oligosaccharides (Ballou 1990).

To assess the permeability of yeast cells were washed twice with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.8), concentrated 200 times and TPPCl was added a final concentration of 3×10^{-7} M (Fig. 23.1). After 30 min of incubation at 30 °C cells were precipitated and the supernatant was used for measuring residual TPP^+. 100 µL of the supernatant was added to 200 µL of TE buffer (with 3×10^{-7} M TPPCl), with the TPP^+ selective combined electrode immersed. The electrode potential drift was estimated with a Hanna pH213 ion meter in magnetically stirred solution, and the quantity of TPP^+ absorbed by yeast cells was calculated (Zimkus and Chaustova 2004). According to Ballarin-Denti et al. (1994) the distribution of TPP^+ ions—measured as plasma membrane voltage—does not reach the equilibrium in intact *S. cerevisiae* cells within

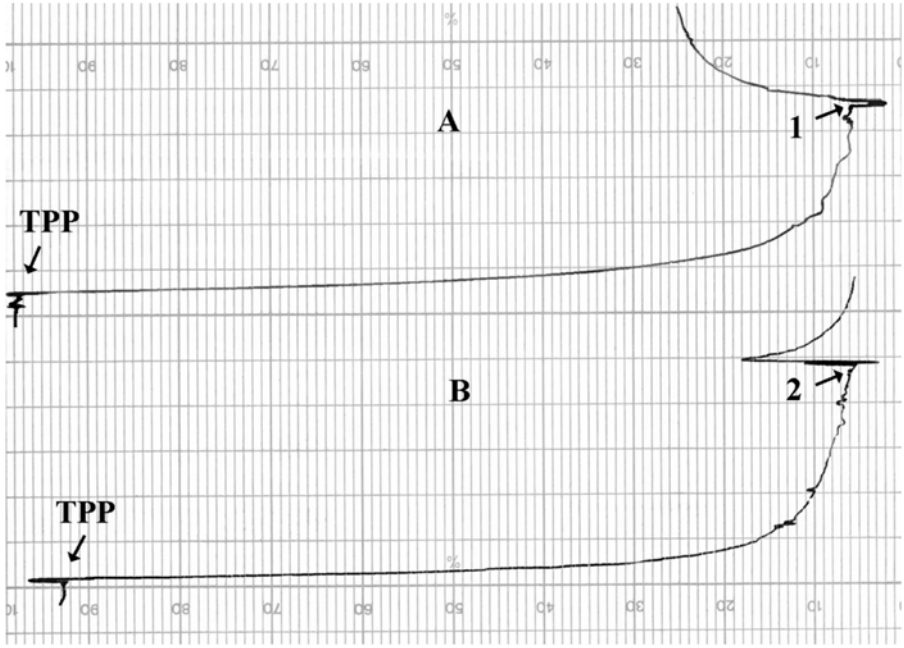

Fig. 23.1 The uptake of TPP^+ ions by *S. cerevisiae* cells represented as decrease in TPP^+ ions concentration after the addition of cell to incubation medium. *Arrows* 1 and 2 indicates the time samples were added. (**a**) Supernatant of yeast cells after 30 min. incubation with TPP^+ ions; (**b**) control aliquot of the medium without incubation with yeast cells. Calibration was performed by increasing the concentration of TPP^+ ions from 2×10^{-7} to 3×10^{-7} M

several minutes. To reach steady-state 0.5–2 h incubation is needed, dependent on the strain (Ballarin-Denti et al. 1994). In our hands, we observed the maximum of accumulated TPP^+ ions after 30 min; longer incubations did not cause significant increase in amount of accumulated TPP^+ ions.

The concentration of remaining TPP^+ ions in the supernatant was estimated after yeast cells incubation (Table 23.1). The amount of lipophilic cations accumulated in strains with a defective cell wall, as SEY6210 (*kre1*), (*kre2*), (*kre6*) and XCY42-30D (*mnn1*), was different and dependent on cell wall structure properties (Table 23.1) (Zimkus and Chaustova 2004). The mutations influencing yeast 1,6-glucan biosynthesis enhanced the permeability of the lipid-soluble TPP^+ ions. SEY6210 (*kre1*) and SEY6210 (*kre6*) absorbed TPP^+ ions 1.7 and 1.5 times more effectively than the parental SEY6210 strain. Strain SEY6210 (*kre2*), defective in α1,2-mannosyl transferase involved in protein *O*-glycosylation (Hausler et al. 1992), caused TPP^+ ions accumulation up to 170 %. A moderate increase in TPP^+ accumulation was observed in XCY42-30D (*mnn1*).

Table 23.1 Transformation efficiency and permeability properties of yeast *S. cereavisiae* strains with defects in cell wall structure

Strain	Number of transformants per 10 μg DNA	TPP^+ ions accumulated as millimoles of TPP per milligram of yeast protein 10^{-9}
SEY6210 (wild type)	$(226\pm22)\times10^2$	7.132 ± 0.366
SEY6210 (*kre1*)	$(174\pm19)\times10^2$	12.357 ± 1.342
SEY6210 (*kre2*)	$(46\pm8)\times10^2$	12.600 ± 0.576
SEY6210 (*kre6*)	$(77\pm6)\times10^2$	10.460 ± 1.168
XCY42-30D (*mnn1*)	$(44\pm8)\times10^2$	8.053 ± 0.621
LB3003-JAa (*mnn9*)	$(43\pm14)\times10^2$	ND

ND not determined

β-1,6-D-glucan is the central molecule that keeps together other components of the cell wall, including β-1,3-D-glucan, mannoprotein, and part

of the chitin (Kollar et al. 1997; Smits et al. 1999, 2001). β-1,6-D-glucan and chitin both are responsible for rigidity of the cell wall and are defining its morphology and shape (Zlotnik et al. 1984; De Nobel et al. 1989, 1990; Ruiz-Herrera 1992). Thus, it is not surprising that defects in β-1,6-d-glucan and mannan formation interfere with cell wall assembly and have severe effects on accumulation of TPP^+ cations. These results point to the critical role of β-1,6-D-glucan and mannoprotein in the barrier properties of the cell wall. These are in good agreement with other data showing that the external protein layer, the *N*-linked side-chains of mannoproteins in particular, determines the permeability of the yeast cell wall (Zlotnik et al. 1984; De Nobel et al. 1989, 1990). The layered structure of the cell wall, being a general phenomenon in yeast, modifies the surface properties such as hydrophobicity, electrical charge, sexual mating, and porosity (Orlean 1997; Lu et al. 1995; Klis 1994; Klis et al. 2002, 2006; Zlotnik et al. 1984; De Nobel et al. 1990, 2000).

Yeast transformation was carried out treating the cells with Li^+ cations (Ito et al. 1983). Plasmid pT11 (4,0 kb, multi-copy, containing the bacterial plasmid pUC9 and yeast gene *TRP* and a part of yeast 2 μm plasmid) was used. Mutations in the cell wall of the recipient *Saccharomyces cerevisiae* cells changed the ability of the yeast cells to be transformed and resulted in a lower number of transformants (Table 23.1). The altered cell wall composition due to the *kre1* mutation caused a decrease in the number of transformants (about 20 %) as compared to the parental SEY6210 strain (Chaustova 2000). Defects in both glucan types change the cell wall assembly and have shown severe effects on the cells' ability to absorb DNA. The number of transformants of *kre6* cells was about 77×10^2 transformants/10 μg DNA (Table 23.1). The *kre2* mutation caused some structural changes in the mannan–protein complex (Hausler et al. 1992; Hill et al. 1992; Ballou 1990). The strains with *mnn* mutations as *kre2, mnn1 and mnn9* turned out to be not highly effective in the transformation; the number of transformants was about 50×10^2 transformants/10 μg DNA (Table 23.1). Kawai et al. (2004) also noted this in mutants with defects in *N*-linked glycosylation (*mnn9 and och1*), *O*-linked glycosylation (*kre2*), and phosphomannosylation (*mnn6*).

Cell wall density, thickness, and structure are essential not only for the survival of fungal cells but also could be factors of major importance during plasmid DNA transfer. The cell wall represents a complex structure of cross-linked glucans, mannoproteins, and chitin. Glucose residues are linked to other glucose molecules through $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 6)$ linkages and to *N*-acetylglucosamine via $\beta(1 \rightarrow 4)$ bonds (Kollar et al. 1997; Lipke and Ovalle 1998; Klis et al. 2002, 2006; Lesage and Bussey 2006). The mannoproteins of *S. cerevisiae* are the most highly exposed cell wall molecules, which are extensively *O*- and *N*-glycosylated. They are densely packed and have significant influence on cell wall permeability (Ballou et al. 1990).

Yeast *S. cerevisiae* transformation is a multistage process, consisting of plasmid DNA absorption on the cell surface to the next penetration into cells. We assume that mutations in the synthesis of carbohydrates can change the structure of the cell wall, maybe it becomes loosened and/or less elastic. All the changes in cell wall structure can affect the binding or absorption of plasmid DNA to the cell wall and it is causing the changes in the efficiency of transformation. In our case the transformation efficiency was reduced. In contrast, mutations in the synthesis of carbohydrates of cell wall evoke an increase the passage of TPP through the cell wall, suggesting accumulation of lipophilic cations associates rather with cell wall permeability and membrane potential.

23.3 Lithium Effect on the Permeability of Yeast *Saccharomyces cerevisiae* Cells

Li^+ ions are eight times more effective in inducing competence for yeast cells than some other cations tested (Na^+, K^+, Rb^+, Cs^+) (Ito et al. 1983). Transformation efficiencies with LiCl or lithium

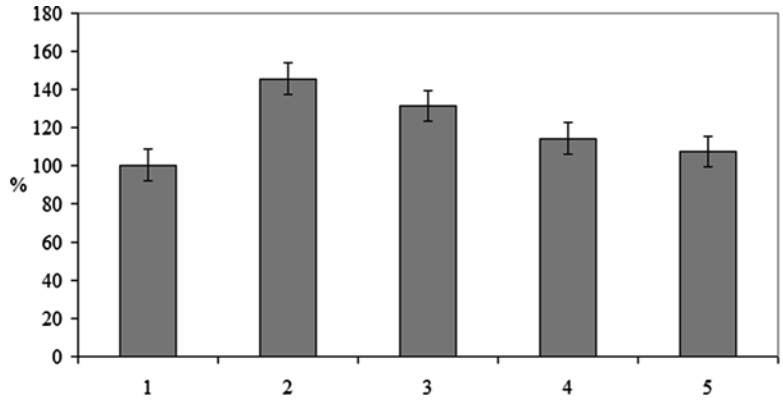

Fig. 23.2 Accumulation of TPP^+ ions by *Saccharomyces cerevisiae* SEY6210. 1: Control, 2: 0.1 M lithium acetate, 3: 0.1 M LiCl, 4: 0.1 M NaCl, 5: 0.1 M sodium acetate. Values are the average ± standard errors of three independent experiments (100 % = 27 nmol TPP^+/mg yeast protein)

acetate exceeded that obtained by the conventional protoplast method (Hinnen et al. 1978). Ca^{2+}, which induces competence in *E. coli* cells, has not been effective on yeast cells. Li^+ ions enhanced the transformation of intact cells, but no effect on transformation of protoplasts was observed, implying that Li^+ ions facilitated the transfer of DNA through the cell wall (Hayama et al. 2002).

The effect of various salts of Li^+ and/or Na^+ cations on permeability of *S. cerevisiae* cells was examined by TPP^+ ions accumulation (Fig. 23.2). Yeast cells incubated without cations were used as control and they accumulated 27 nmol of TPP^+ per mg of yeast protein. Incubations with lithium acetate and LiCl increases TPP^+ accumulation by 1.5 and 1.3 times. The amount of TPP^+ ions accumulated after the treatment with Na^+ ions was lower: 115 %. (Zimkus et al. 2006).

As treatment of intact *S. cerevisiae* cells with Li^+ ions increased the permeability to TPP^+ ions, this suggests certain structural changes in the yeast. We do not exclude the possibility that other effects could be induced by Li^+ ions. There is evidence of the effect of Li^+ ions on the structure of DNA. According to molecular dynamic studies, Li^+ ions bind to the phosphate oxygen atoms of DNA and are capable to form stable ion pairs without disrupting the water structure around DNA (Lyubartsev and Laaksonen 1998; Sundaresan et al. 2006). Additionally, it is known that high concentrations of Li^+ ions can induce the formation of liquid crystalline (LC) phases of the DNA structure, due to the extremely high hydration radius of Li^+ ions (Sundaresan et al. 2006). Differences between Li^+ ions and other alkali metal ions can be explained by a higher number of water molecules in the hydration sphere of lithium (Sundaresan et al. 2006). The unusual stability observed for the liquid LC of DNA in the presence of Li^+ ions might be caused by the complexation behavior of Li^+ ions to DNA and its water retaining capability. It is plausible, that such complex of DNA and Li^+ ions is thereby more suitable for transformation process.

23.4 Spectroscopic Study of Yeast *Saccharomyces cerevisiae* Cell Wall Structure

Fourier transform infrared spectroscopy (FT-IR) method was applied to characterize the changes in yeast cell wall structure. An infrared spectrum of complex biological materials does not only describe the composition of cell, but also provide a number of specific bonds that are sensitive to structural or conformational changes. The importance of FT-IR spectroscopy is that it allows real time in vivo detection of dynamic interface events in near-physiological conditions (Naumann 2000; Naumann et al. 1991, 1995;

Helm and Naumann 1995; Orsini et al. 2000; Galichet et al. 2001). FT-IR has been used for the differentiation of bacteria as well as filamentous fungi and yeasts (Fischer et al. 2006; Kümmerle et al. 1998; Mariey et al. 2001; Sandt et al. 2003; Santos et al. 2010; Wenning et al. 2002). Preliminary data indicates that eukaryotic microorganisms such as yeasts may also be identified by FT-IR (Henderson et al. 1996). FT-IR spectroscopic demonstrated high sensitivity and simplicity in measuring cell wall features of yeast strains possessing different cell wall architecture. The proportion of these components may vary between exponentially growing and stationary phase cells, between parental and mutated lines, during the cell cycle or in response to environmental conditions such as nutrient and oxygen availability, temperature, and pH (Aguilar-Uscanga and François 2003). FT-IR has been extensively used in the past years for studying microbial surfaces, identification and classification of microorganisms (Siebert 1995; Ojeda et al. 2008; Helm and Naumann 1995; Orsini et al. 2000; Karreman et al. 2007; Santos et al. 2010).

The effect of Li^+ ions on the molecular structure of yeast cell walls was studied with two *S. cerevisiae* strains: p63-DC5 (wild-type) and XCY42-30D (*mnn1*), with some structural changes in the mannan–protein complex (Raschke et al. 1973).

S. cerevisiae mannan–protein has a linear $\alpha(1\rightarrow6)$-linked backbone with side chains of $\alpha(1\rightarrow2)$- and $\alpha(1\rightarrow3)$-linked mannose units. In *S. cerevisiae* mannoproteins contribute to the regulation of the cell wall porosity, and therefore control both the secretion of proteins and the entrance of macromolecules from the environment. The cell wall mannoproteins of *S. cerevisiae* are the most highly exposed cell wall molecules, which are extensively *O*- and *N*-glycosylated. *O*- and *N*-oligosaccharides of yeast contain mannosyl phosphate residues that confer a net negative charge to the cell wall (Ballou et al. 1990).

The transformation efficiency of both strains, p63-DC5 and XCY42-30D (*mnn1*), was induced by Li^+ ions, though to a lesser extent for XCY42-30D (*mnn1*) (29 %) (Zimkus et al. 2013).

Table 23.2 Comparative data of transformation efficiency of *S. cerevisiae* p63-DC5 and XCY42-30D (*mnn1*) strains; number of transformants per 10 μg DNA

Strains	Number of transformants		Viability, cells/mL
	0.1 M LiCl	0.1 M NaCl	
P63-DC5	$(233\pm46)\times10^2$	$(44\pm5)\times10^2$	2.0×10^8
XCY42-30D (*mnn1*)	$(65\pm4)\times10^2$	0	2.0×10^8

Most likely, the lower transformation efficiency XCY42-30D (*mnn1*) is due to the structural particularities of the outer layer of mannan–protein complex of the cell wall.

Transformation experiments were also carried out with 0.1 M NaCl to control the possible influence of ionic strength on the spectral bands. The transformation efficiency of p63-DC5 with 0.1 M NaCl was approximately 20 % of that obtained with 0.1 M LiCl, while no transformants were observed after the treatment of XCY42-30D (*mnn1*) with 0.1 NaCl (Table 23.2). These results are in agreement with those of Ito et al. (1983).

The FT-IR spectra of yeast *S. cerevisiae* p63-DC5 and XCY42-30D (*mnn1*) strains are shown in Fig. 23.3. The spectral assignments were done on the basis of the literature data (Laidiga et al. 2000; Misiūnas et al. 2008). Absorption of mannans and glucans—the principal constituents of cell wall—are observed in the spectral region between 970 and 1,185 cm^{-1} (Misiūnas et al. 2008). It should be noted that bands from the O–P–O group and the phosphate ester C–O–P stretch (Casal et al. 1973) also appear in this region, so detailed band assignments are difficult. However, it is generally accepted that the content of glucan and mannan in the yeast cell wall is much higher than that of phosphates (Kapteyn et al. 1999). The definite changes of $\beta(1\rightarrow3)$ glucan bands positions depending on the strain were determined, whereas the position of both mannan bands was practically unchanged (Table 23.3).

The band positions of the vibrations by pyranose rings of the carbohydrate groups of p63-DC5 strain are practically not influenced by Li^+ ions. Treatment of XCY42-30D (*mnn1*) strain Li^+ ions shifts the glucans band positions by 1–3 cm^{-1}.

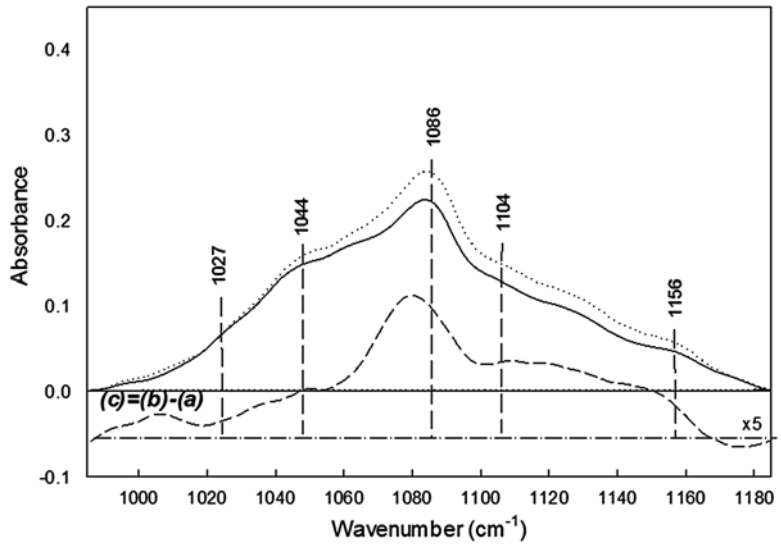

Fig. 23.3 Normalized FT-IR spectra of *S. cerevisiae* p63-DC5 (*solid line*) and XCY42-30D (*mnn1; dotted line*) strains in the 10 mM Tris–HCl, pH 8.0 in the frequency region of 900–1,180 cm^{-1}. Difference spectra (*dashed line*) are shown

Table 23.3 Wavenumbers (cm^{-1}) of the FT-IR carbohydrate bands of yeast *S. cerevisiae* P63-DC5 and XCY42-30D (*mnn1*) cells and the shifts (cm^{-1}) of the bands induced by the treatment with 0.1 M LiCl or 0.1 M NaCl

p63-DC5 strain		XCY42-30D (*mnn1*) strain		
Wavenumber (cm^{-1})	Wavenumber (cm^{-1})	Wavenumber (cm^{-1})	Wavenumber (cm^{-1})	
LiCl added	NaCl added	LiCl added	NaCl added	Assignment
971	971	970	970	Mannan band (C–O–C, C–C, and C–OH stretching of pyranose ring), O–P–O, and C–O–P stretching
1,027	1,026	1,028	1,027	β(1 → 4) glucan band (C–O–C, C–C, and C–OH stretching of pyranose ring), O–P–O, and C–O–P stretching
1,044	1,044	1,045	1,042	Mannan band (C–O–C, C–C, and C–OH stretching of pyranose ring), O–P–O, and C–O–P stretching
1,086	1,086	1,086	1,084	β(1 → 3) glucan band (C–O–C, C–C, and C–OH stretching of pyranose ring), O–P–O, and C–O–P stretching
1,104	1,104	1,104	1,106	β(1 → 3) glucan band (C–O–C, C–C, and C–OH stretching of pyranose ring), O–P–O, and C–O–P stretching
1,156	1,156	1,154	1,152	β(1 → 3) glucan (C–O–C, C–C, and C–OH stretching of pyranose ring), O–P–O, and C–O–P stretching

In the difference spectrum (dashed line) positive peaks at 1,104 cm^{-1} and especially at 1,086 cm^{-1} show that the quantity of β(1 → 3) glucans is higher in XCY42-30D (*mnn1*) strain (Fig. 23.3). The characteristic band at 1,045 cm^{-1} illustrates that the content of mannans is only slightly increased in XCY42-30D (*mnn1*) strain.

XCY42-30D (*mnn1*) is defective in the synthesis of the α-(1 → 3)-mannosyl transferase (*mnn1*), which is involved in the addition of the

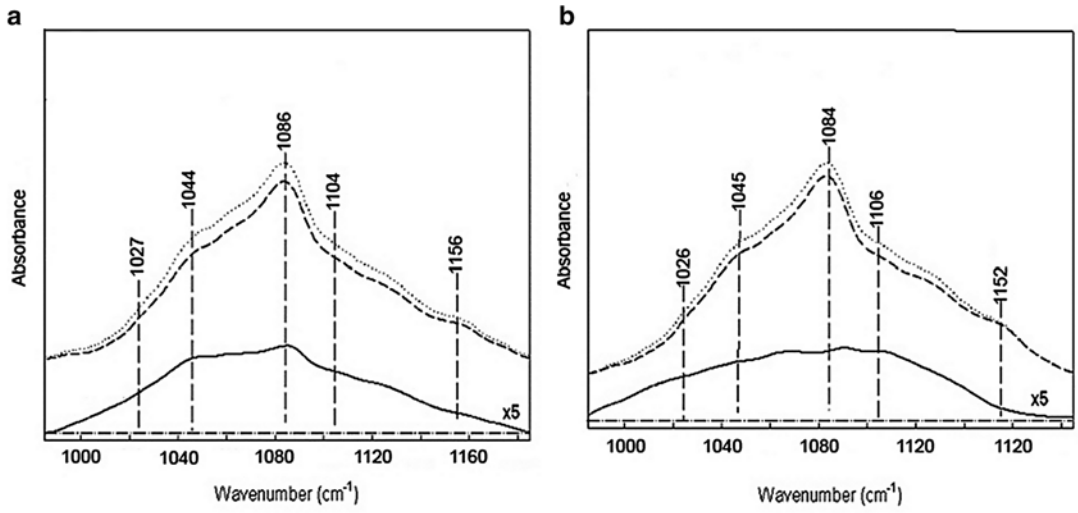

Fig. 23.4 FT-IR spectra of (A) *S. cerevisiae* p63-DC5 in 0.1 M LiCl buffer (*dotted line*), and 0.1 M NaCl buffer (*dashed line*); (B) *S. cerevisiae* XCY42-30D (*mnn1*) in 0.1 M LiCl buffer (*dotted line*) and in 0.1 M NaCl buffer (*dashed line*). Difference spectra (LiCl buffer minus NaCl buffer; *solid line*) are also shown

terminal α-(1 → 3)-linked mannose unit to form the mannotetraose side chain. In the cell wall of XCY42-30D (*mnn1*) α(1 → 6) connected mannoses molecules dominate, and may exist as chains of two units (Raschke et al. 1973). Since the mannans of p63-DC5 have structures with three or more mannose units linked by α(1 → 3) and the α(1 → 3) bonds are dominant, we assume that the decrease in α(1 → 3) linked mannose in XCY42-30D (*mnn1*) cells can be compensated by increasing the synthesis of β(1 → 3) linked glucans, which determine cell wall rigidity and stability. Thus, the cell wall of XCY42-30D (*mnn1*) cells may be thicker than the cell wall of p63-DC5 strain and this is presumably the factor responsible for lowering the transformation efficiency.

Li^+ ions did not change the interactions of carbohydrate groups in p63-DC5, rather the cell wall of XCY42-30D (*mnn1*) strain underwent conformational changes affected the ability of DNA to penetrate into the yeast cell (Fig. 23.4). As Li^+ ions could not change the content of main components of the cell wall, but could modify the surface of the mannan layer and as a result the surface of the yeast cells could become more porous and this could facilitate the penetration of DNA through the cell wall. These findings are in agreement with results presented by Chen et al. (2008), which observed the changes in the surface of intact yeast cells affected by Li^+ ions. Atomic force microscopy showed that the cell surface became much rougher and wrinkled after incubation of yeast cells with Li^+ ions.

23.5 Competence of Yeast *Saccharomyces cerevisiae* Determined by Metabolic State and Cell Cycle

For successful fungal transformation exogenous DNA must pass through the cell wall and plasma membrane and then it be delivered into a cytosol. Since there are no conventional concepts to explain the mechanism of DNA penetration, in many cases the selection of treatments and their combinations are carried out empirically. It was shown that the cell capacity to become competent reached the maximum between the early- and mid-log phases, rapidly decreasing after mid-log phase. DNA uptake is induced in yeast cells only in the early log phase (Hayama et al. 2002; Kawai et al. 2010). According to a well-known and

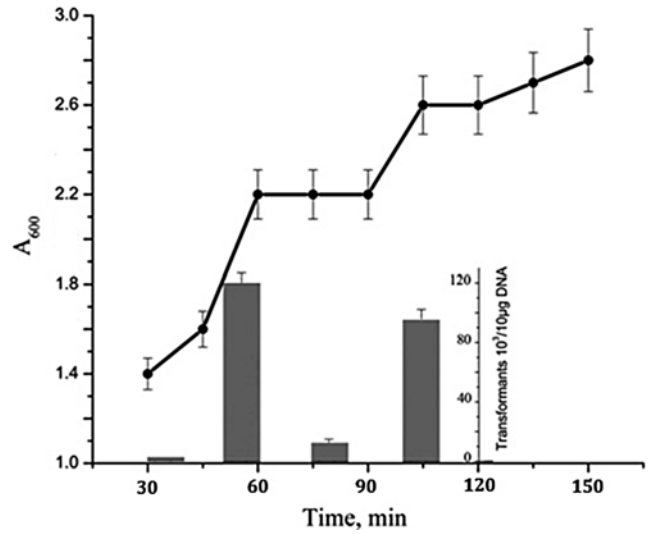

Fig. 23.5 Variability of transformation efficiency of *Saccharomyces cerevisiae* p63-DC5 cells by plasmid pL3 during exponential growth. The data represents the average values ± SD of three independent experiments

widely applied method proposed by Gietz et al. (1992) the increase in transformation efficiency was achieved after the dilution of the overnight pre-cultures, allowing the cells to grow and complete at least two generations. This increased the transformation efficiency up to 1.2×10^6/10 μg of DNA.

Significant changes in the transformation efficiency of during exponential growth of yeast *S. cerevisiae* p63-DC5 strain were observed when the culture growing overnight was re-cultivated (Chaustova and Jasaitis 1994). The efficiency of the process in separate experiments varied from maximal to minimal, up to the complete absence of transformants. The transformation efficiency in samples evaluated at different intervals of growing culture is presented in Fig. 23.5. As it is seen from this figure, the transformation efficiency ranges from $1.0–2.0 \times 10^2$ to $1.2–1.5 \times 10^4$/1 μg of plasmid DNA. Considerable changes of transformation efficiency during the growth of the synchronized culture indicate the dependence on certain changes in the phases of cell cycle.

The cell cycle is the sequence of events by which a growing cell replicates all of its components and divides them into two daughter cells (Nasmyth 2001). Between one cell division and the next, all essential components of the cell must be duplicated. The processes of DNA replication and sister chromatid separation occur in temporally distinct phases of the eukaryotic cell cycle. These are known as S-phase (DNA synthesis) and M-phase (mitosis), In general, S- and M-phases are separated by two gaps, known as G1 and G2 (Brewer et al. 1984).

The relationship between cell cycle and transformation efficiency was studied using yeast cultures synchronized by specific agents. To p63-DC5 arrested in S- or M-phase, 10 mM hydroxyurea or 1 μg/mL of colchicine were used, respectively (Venturi et al. 2000; Marenzi et al. 1999; Cohen et al. 1981; Kamei 1995). Ninety percent of the cells arrested in S-phase through hydroxyurea were budded (Chaustova and Zimkus 2004; Chaustova et al. 2008). The number of transformants obtained with yeast cells in this phase was much higher than with those from an asynchronous culture: 1.4×10^5 transformants per 1 μg DNA, which is 267 % higher (Table 23.4). The microtubule-disrupting agent colchicine induces mitotic arrest in the M-phase. The number of transformants from cells treated with colchicine was considerably lower and reached only 2.4×10^4 transformants (45 %). As transformation efficiency of S-phase cells was about six times higher than that of M-phase cells. It is possible to conclude that the yeast cells in the S-phase of growth have the highest capability of taking up exogenous DNA.

Table 23.4 Comparison of the transformation efficiency of asynchronous and synchronization cells of *Saccharomyces cerevisiae* p63-DC5 strain

Phase of cells growth	Number of transformants per 10 μg of plasmid DNA	(%)
Asynchronous (control)	$(540 \pm 20) \times 10^2$	100
S-phase	$(1{,}440 \pm 40) \times 10^2$	267
M-phase	$(240 \pm 20) \times 10^2$	45

The plasmid pL3 (7.7 kb, multi-copy, containing a 2.2-kb PstI fragment carrying the *LEU2* gene, a 2.2-kb EcoRI fragment of the 2 μm plasmid DNA, and the sequences of bacterial plasmid pBR327; Sasnauskas et al. 1991) was employed in transformation experiments

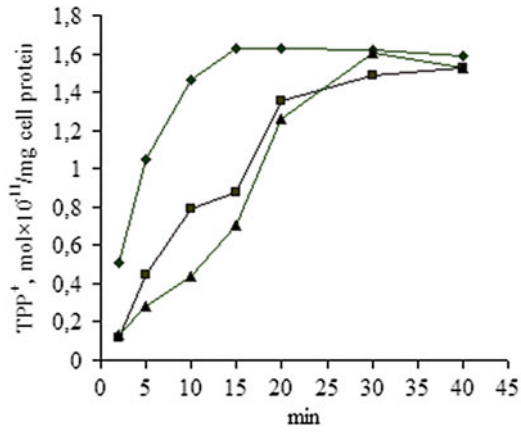

Fig. 23.6 Kinetics of TPP^+ ions accumulation by yeast *S. cerevisiae* p63-DC5 strain. *Filled diamond*: synchronous yeast cells in S-phase; *filled square*: synchronous yeast cells in M-phase; *filled triangle*: asynchronous yeast cells; Means of the data of 3 experiments are presented

Thus, high transformation efficiency is not a simple function of cell growth but is dependent on cell cycle. The relation between cell cycle and some cell functions is well-known (Caro et al. 1998; Marenzi et al. 1999; Rodriguez-Pena et al. 2000; Smits et al. 2001; Klis et al. 2006). Marenzi et al. (1999) showed high gene expression when S-phase synchronized L929 mouse fibroblast cells were used for transfection and the presence of specific cell wall enzymes.

Several studies have shown that the yeast cell wall is not a static shield but a highly dynamic structure that can be changed accordingly to the physiological needs of the cell. (De Nobel et al. 1990, 2000; Popolo et al. 2001; Cid et al. 1995; Klis et al. 2006).

The results presented demonstrate that the efficiency of *S. cerevisiae* plasmid transformation was influenced and changed during the cell cycle. One of the reasons may be related to cell wall properties and the morphogenetic processes of yeast during the S-phase which include: secretion of other materials to the surface of the bud and localization of new growth to the tip of the bud.

During the cell cycle, the cell wall becomes more flexible at the point of bud emergence where the growth takes place. The rapid growth of buds suggests that the events steering the development of such an apparently rigid structure may allow certain flexibility, at least in growing areas, without altering the protective function of the cell wall (Pringle et al. 1986; Pringle 1991; Rodriguez-Pena et al. 2000). In this regard, cell wall of *S. cerevisiae* exhibits variations in porosity during growth and cell division. Maximum porosity is observed during bud growth where the cell wall is in a more flexible, expanded state as compared with stationary phase cells (de Nobel et al. 1990, 2000; Spellman et al. 1998; Popolo et al. 2001).

The changes in the morphogenetic process and the integrity of the cell wall complex occurring in the S-phase may affect permeability of yeast cells so that DNA could penetrate it. Permeability properties of the yeast cells were estimated by measuring the accumulation of the tetraphenylphosphonium ions (Zimkus and Chaustova 2004; Rotenberg 1997; Ballarin-Denti et al. 1994). Intact *S. cerevisiae* cells do not reach TPP^+ ions equilibrium distribution within a few minutes as was observed for spheroplast. To reach steady-state for some yeast strains this takes from 15 to 120 min (Ballarin-Denti et al. 1994; Rotenberg 1997). We observed maximum TPP^+ accumulation after 30 min (Zimkus and Chaustova 2004).

The kinetics of TPP^+ ions accumulation for p63-DC5 cells in S- and M-phases are different (Fig. 23.6). Yeast cells in both phases as well as asynchronised cells reach steady-state within

30 min at 30 °C. But the amount of TPP^+ ions accumulated at various time was quite different. The rate of TPP^+ ions accumulation by cells in S-phase was much higher in the first 10 min of incubation and achieved a steady-state after 15 min. While M-phase and asynchronised cells follow more similar kinetics, the S-phase cells already reach steady state levels after 10–15 min.

The efficient transformation of yeast cells in S-phase seems to be comparable with the efficiency of genetic transformation of *B. subtilis* in the state of competence (Dubnau 1999). The development of competence and expression of the uptake machinery is well-known phenomenon in bacteria (Dubnau 1999; Dreiseikelmann 1994; Elkins et al. 1991; Hakenbeck 2000). The best characterized naturally transformable bacteria are the Gram-negative species *Haemophillus influenza* (Elkins et al. 1991), the Gram-positive *Bacillus subtilis* (Dubnau 1999; Dreiseikelmann 1994) and *Streptococcus pneumoniae* (Hakenbeck 2000). However, only a minor subpopulation, never exceeding 20 %, of the bacterial cells in a growing culture develops the competence.

The possibility to develop natural competence for *S. cerevisiae* cells was proposed by Nevoigt et al. (2000). In this case the genetic transformation could be achieved only artificially under starvation conditions when 1 M glucose and 1 M fructose were used, furthermore, a much higher concentration of DNA was needed.

These and our results suggest that natural competence is occurred not only between bacterial species but also in eukaryotic yeast cells. The state of the exponential growing yeast cells, which can be named natural competence, was associated with the S-phase of cell cycle, when budded cells dominate in growing culture, and changes in cell wall structure occur.

To achieve high transformation efficiency, a number of factors should be taken into account, including selection of suitable methods, gene expression system, transformation process optimization, etc. The cell cycle of yeast cells is one of the important factors.

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Part IX

Exogenous DNA: Integration of DNA

Recombination and Gene Targeting in *Neurospora*

24

Keiichiro Suzuki and Hirokazu Inoue

24.1 Introduction

24.1.1 Exogenous DNA Integration in Yeast and Filamentous Fungi

Exogenous DNA-mediated genome editing is an important method to investigate gene structure and function through gene cloning. Historically, there have been some attempts to transform fungi in the 1970s, including the pioneering work by Hinnen et al. (1978) describing the transformation experiment in which a chimeric ColE1 plasmid carrying the yeast *leu2* gene was used to transform a yeast *leu2*$^{-}$ strain to *LEU2*$^{+}$. The transforming sequences integrated not only into the *leu2* region but also in several other chromosomal locations by recombination (Hinnen et al. 1978). Orr-Weaver et al. (1981) first showed that linear or gapped-linear plasmid DNA is integrated at a high frequency into homologous sites in yeast chromosomes, meaning that DNA ends are highly recombinogenic and interact directly with homologous sequence. The DNA double-strand break (DSB) repair-deficient mutation *rad52-1* blocked the integration of linear and gapped-linear DNA but not circular DNA, indicating that the DNA DSB repair system is involved in the integration of linear plasmids (Orr-Weaver et al. 1981). Also, linear DNA with short homologous regions (30–50 bp) at both ends can be integrated with high efficiencies (>70 %) via homologous recombination (HR) at the homologous site, suggesting that the exogenous DNA is predominantly integrated in chromosomal DNA via HR and that a short homology sequence is enough for targeted integration in *Saccharomyces cerevisiae* (Guldener et al. 1996).

In contrast to *S. cerevisiae*, integration of exogenously introduced DNA into chromosomes occurs at random sites, and the frequency of homologous integration (HI) is very low in filamentous fungi as well as in higher organisms. The process of exogenous DNA integration is carried out through DSB repair mechanisms. There are two major DSB repair pathways, HR and nonhomologous end-joining (NHEJ; Critchlow and Jackson 1998). HR requires an undamaged homologous sequence to serve as a template for the repair of DSBs. Conversely, NHEJ does not require homologous sequence between two DNA double-stranded ends. The NHEJ process is mediated by the Ku70-Ku80 heterodimer, the DNA ligase IV-Xrcc4 complex, which is specific to the NHEJ repair pathway, and other accessory proteins. The Ku70-Ku80 heterodimer

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binds to DNA ends tightly, a key step in the NHEJ repair pathway. The choice of HR and NHEJ depends on cellular status, including cell cycle and tissue specificity and the nature of DSB ends (Symington and Gautier 2011). Although the DSB repair mechanisms have been conserved through evolution, filamentous fungi preferentially use the NHEJ pathway for DSB repair.

24.1.2 DSB Repair and Exogenous DNA Integration in *Neurospora*

Recombination is essential for maintenance of genome integrity and exchange of genetic information. In the model fungus *Neurospora crassa*, meiotic products of a cross are all recovered and arranged linearly in ascus. Therefore, meiotic recombination events between homologous chromosomes have been studied extensively by genetic and cytological methods (Perkins and Barry 1977). However, fungal nuclei in the vegetative phase are usually haploid and recombination events in mitosis occur during sister chromatid exchange. Therefore, there are some difficulties in studying mitotic recombination. Transformation experiments that introduce DNA segments to recipient cells can make various DNA–DNA interactions containing partial diploid or integration of introduced DNA into genomic DNA. Transformation is therefore useful to study recombination mechanisms in vegetative cells, and especially to analyze DSB repair of DNA.

Many DNA repair mutants that show high sensitivity to mutagens were isolated and characterized in filamentous fungi (Schroeder et al. 1998). At the end of the twentieth century, some DNA repair genes were cloned using cosmid DNA libraries in *N. crassa*, and *mei-3, mus-11* and *mus-25*, genes homologous to *S. cerevisiae RAD51, RAD52*, and *RAD54*, respectively, that play central roles in HR, were identified (Sakuraba et al. 2000; Handa et al. 2000; Hatakeyama et al. 1995). In the beginning of the twenty-first century, the entire genome sequence of *N. crassa* was reported (Galagan et al. 2003), since then, up until today, the genomes of over 100 species of fungi have been sequenced and published. Post-genome project, a functional genomics venture was planned in *N. crassa* (Borkovich et al. 2004; Dunlap et al. 2007), and subsequently, efficient procedures to disrupt genes through targeted gene replacement were sought for high-throughput functional genomics. However, although gene disruption by HR is useful, it is laborious and time-consuming since targeted gene integration is rare in *Neurospora*.

The study of DNA DSB repair machinery in *S. cerevisiae* elucidated that there are at least four major DSB repair pathways; HR, NHEJ, single-strand annealing (SSA), and microhomology-mediated end-joining (MMEJ) (Heyer et al. 2010; Ma et al. 2003; Symington and Gautier 2011; Yu and Gabriel 2003; Fig. 24.1a). Based on DSB repair machinery, it was expected that overexpression of genes related to HR, such as *RAD51, RAD52,* and *RAD54* would raise HI rates of exogenous DNA in fungi. However, the anticipated results were not seen. As breakthrough experiments to raise HI rate, *N. crassa* strains defective in NHEJ, *mus-51, mus-52*, or *mus-53*, that are homologs of human *KU70*, *KU80*, or *Lig4*, respectively, were used as recipient cells in transformation experiments. NHEJ-defective *N. crassa* mutants do not show severe defects in vegetative growth, morphology, or crossing during the sexual cycle, though they are moderately sensitive to mutagens that produce DSBs. When DNA segments exceeding 1 kb in length, at both the 5′- and 3′-region of the target gene of drug-resistant genes, were introduced by electroporation, 100 % of the drug-resistant transformants had replacement at the target gene in NHEJ-defective strains, and only 3–20 % in wild type and nearly 0 % in HR-defective mutants

(Ishibashi et al. 2006; Ninomiya et al. 2004). Importantly, the NHEJ mutation is easily eliminated by one cross to a wild-type strain. Altogether, NHEJ-deficient host cells would be a suitable and safe platform for highly efficient targeted gene modification in *N. crassa*.

Further extensive analyses using a series of DSB repair-defective single and double mutants demonstrated that there are at least four exogenous DNA integration pathways in *N. crassa*, which are similar to the DSB repair pathways (Ishibashi et al. 2006; Fig. 24.1b). There are two HI pathways (MEI-3/Rad51 dependent and independent) and two nonhomologous integration (NHI) pathways (MUS-52/Yku80 dependent and independent). Importantly, MUS-53/Dnl4 was proven as an essential factor in NHI pathways. Therefore, exogenous DNA integration occurred only at the targeted site using HR-pathway in *mus-53* mutants.

24.2 NHEJ-Defective Mutants in Other Fungus

Recently, similar findings have been made in various fungi (Table 24.1; Alshahni et al. 2011; Bugeja et al. 2012; Chang 2008; Chang et al. 2010; Choquer et al. 2008; da Silva Ferreira et al. 2006; de Boer et al. 2010; de Jong et al. 2010; El-Khoury et al. 2008; Fang et al. 2012; Fox et al. 2009; Goins et al. 2006; Guangtao et al. 2009; Haarmann et al. 2008; He et al. 2013; Ishidoh et al. 2014; Krappmann et al. 2006; Kuck and Hoff 2010; Lan et al. 2008; Li et al. 2010; Meyer et al. 2007; Mizutani et al. 2008; Nakazawa et al. 2011; Nayak et al. 2006; Poggeler and Kuck 2006; Schorsch et al. 2009; Takahashi et al. 2006; Tani et al. 2013; Ushimaru et al. 2010; Villalba et al. 2008), plants (Nishizawa-Yokoi et al. 2012; Tanaka et al. 2010) and mammals (Bertolini et al. 2009; Fattah et al. 2008; Iiizumi et al. 2008), showing, in almost all cases, that HI rates are highly increased in NHEJ-defective strains. Methods that introduce DNA fragments into recipient cells were different in each organism, such as electric-pulse, spheroplast-fusion, *Agrobacterium*-mediated, and particle-gun methods. The frequency of HI in NHEJ-defective strains ranges from 50 to 100 % in different species, suggesting the presence of another NHI system, different from the Ku/Lig4 system.

In *Neurospora* gene-knockout projects that disrupt all hypothesized genes having open reading frames, *mus-51* or *mus-52* mutations, were used effectively (Colot et al. 2006). Extensive Southern blot analysis of over 600 independent transformants demonstrated that over 98 % of them showed accurate gene replacement, inserting the knockout cassette in the targeted genes, without any ectopic insertion (Colot et al. 2006). Around 10,000 genes have been systematically deleted in *mus-51* or *mus-52* mutant genetic background strains and over 12,000 obtained knockout strains are distributed to researchers through the Fungal Genetics Stock Center (FGSC).

Obtained mutants through HI in NHEJ-deficient strains may have unknown phenotypic effects from NHEJ mutations. To avoid this problem, transient knockdown of Ku70/Ku80/Lig4 via RNA interference and conditional excision by the Cre/lox system have been developed (Nielsen et al. 2008; Szewczyk et al. 2013; Tani et al. 2013).

24.3 Summary

Exogenous DNA is integrated in chromosomal DNA via DSB repair mechanisms: HI mediated by HR and NHI mainly mediated by NHEJ. In filamentous fungi, NHEJ-defective strains are powerful tools for efficient genetic transformation techniques. This method will contribute to broad functional genomics for biological study in filamentous fungi and will enable the generation of novel engineered strains for industrial purposes.

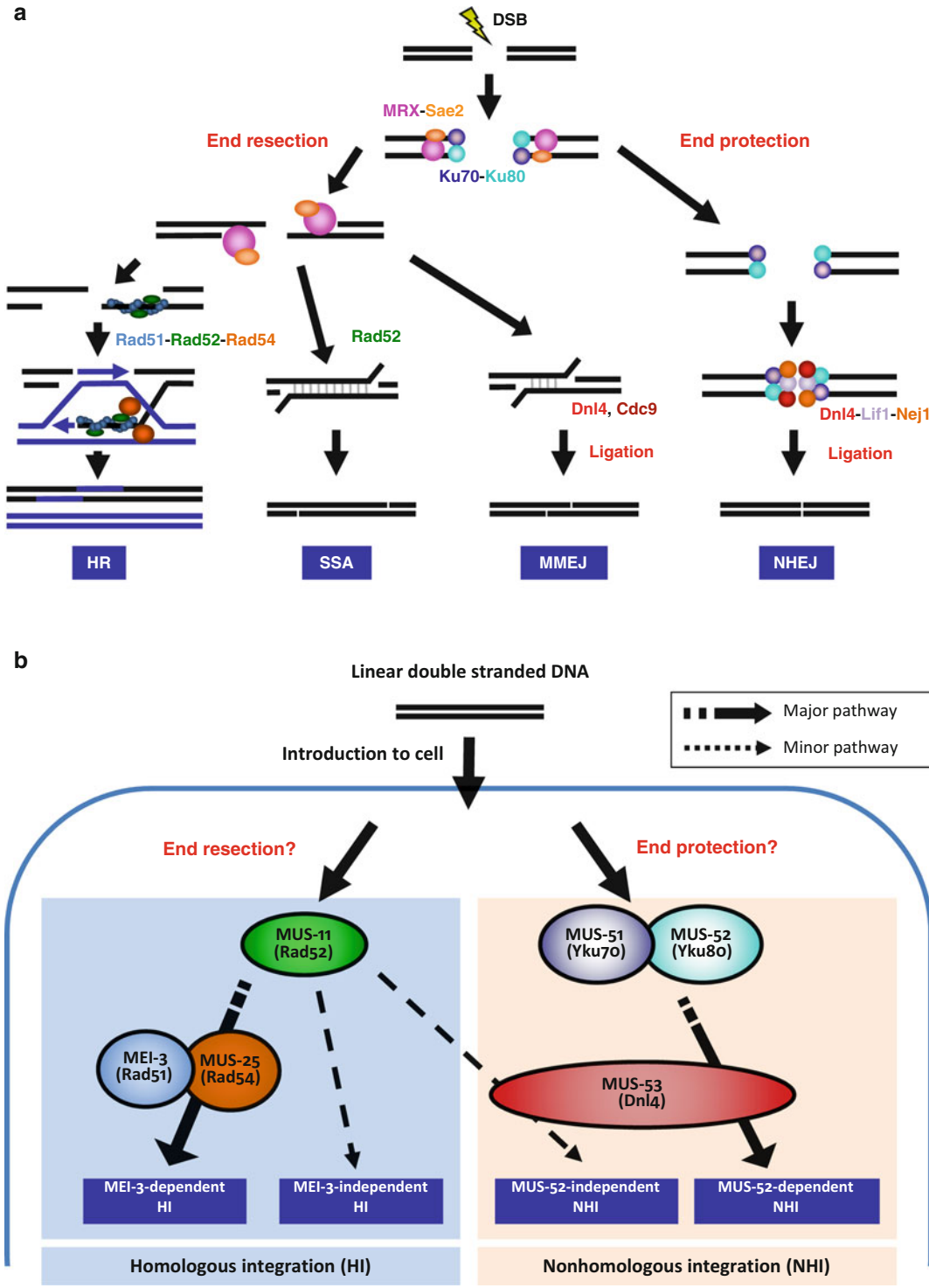

Fig. 24.1 DNA DSB repair and exogenous DNA integration pathways. (**a**) DNA DSB repair pathways in *S. cerevisiae*. DSBs are repaired by at least four different pathways. The NHEJ pathway is initiated by the binding of Ku heterodimer (Yku70 and Yku80) to DSB ends. Ku heterodimer protects double-strand DNA ends from digestion by nucleases and recruits DNA ligase IV (Dnl4) and accessory proteins (Lif1 and Nej1). Dnl4 ligates both

Table 24.1 HI frequency in fungus NHEJ-deficient strains

Organism	Recipient	HI frequency	Reference
Aspergillus aculeatus	Δku80	96 %	Tani et al. (2013)
Aspergillus flavus	Δku70	80–100 %	Chang et al. (2010)
Aspergillus fumigatus	Δku70	100 %	Krappmann et al. (2006)
A. fumigatus	Δku80	80 %	da Silva Ferreira et al. (2006)
Aspergillus glaucus	Δlig4	85 %	Fang et al. (2012)
Aspergillus nidulans	Δku70	92 %	Nayak et al. (2006)
Aspergillus niger	Δku70	80 %	Meyer et al. (2007)
Aspergillus oryzae	Δlig4	100 %	Mizutani et al. (2008)
Aspergillus parasiticus	Δku70	96 %	Chang (2008)
Aspergillus sojae	Δku70	70 %	Takahashi et al. (2006)
Botrytis cinerea	Δku70	Increased	Choquer et al. (2008)
Claviceps purpurea	Δku70	50–60 %	Haarmann et al. (2008)
Colletotrichum higginsianum	Δku70	Highly increased	Ushimaru et al. (2010)
Coprinopsis cinerea	Δku70/Δlig4	Highly increased	Nakazawa et al. (2011)
Cryphonectria parasitica	Δku80	80 %	Lan et al. (2008)
Cryptococcus neoformans	Δku	Highly increased	Goins et al. (2006)
Hypocrea jecorina	Δku70	95 %	Guangtao et al. (2009)
Lecanicillium sp. HF627	Δku80	62 %	Ishidoh et al. (2014)
Magnaporthe grisea	Δku80	80 %	Villalba et al. (2008)
Monascus rubber M7	Δku80	61 %	He et al. (2013)
N. crassa	Δku70/Δku80	100 %	Ninomiya et al. (2004)
N. crassa	Δlig4	100 %	Ishibashi et al. (2006)
Penicillium chrysogenum	Δku70/Δlig4	70 %	de Boer et al. (2010)
Penicillium decumbens	Δku70	100 %	Li et al. (2010)
Penicillium marneffei	Δlig4	Dramatically increased	Bugeja et al. (2012)
Pichia ciferrii	Δlig4	Dramatically increased	Schorsch et al. (2009)
Podospora anserine	Δku70	100 %	El-Khoury et al. (2008)
Schizophyllum commune	Δku80	100 %	de Jong et al. (2010)
Sordaria macrospora	Δku70	100 %	Poggeler and Kuck (2006)
Toxoplasma gondii	Δku80	100 %	Fox et al. (2009)
Trichophyton mentagrophytes	Δlig4	90 %	Alshahni et al. (2011)

Fig. 24.1 (continued) ends of the DSB. The MRX complex (Mre11, Rad50, and Xrs2) and Sae2 displace the Ku complex and inhibit NHEJ. For HR pathways, the 3′-single-stranded DNA (ssDNA) generated by MRX-Sae2 resection is bound by Rad51 proteins, making a fil ment structure; it invades homologous double-stranded DNA together with Rad52, Rad54, and other proteins. Using a homologous region as a template, the DSBs are repaired without any mutations. The 3′-ssDNA is also processed by the Rad52-mediated SSA pathway only when two long repeat sequences flank the DSB site. MMEJ is a Ku-independent end-joining pathway mediated when short (5–25 bp) homologous sequences anneal to both strands. Overhanging bases (flaps) are removed and both ends on the single strand of DNA are connected by Dnl4 and/or Cdc9. (**b**) Exogenous DNA integration pathways in *N. crassa*. Linear exogenous double-strand DNA is introduced in the cells and is integrated into genomic DNA via one of at least four different pathways; two involving MEI-3 (Rad51 homolog)-dependent and -independent HI and two involving MUS-52 (Ku80 homolog)-dependent and -independent NHI. Both NHI pathways, MUS-52-dependent and -independent, require DNA ligase IV (MUS-53). Both HI pathways and MUS-52-independent NHI pathway require Rad52 homolog, MUS-11. The genes in parentheses represent *S. cerevisiae* homologs

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Efficient Generation of *Aspergillus niger* Knock Out Strains by Combining NHEJ Mutants and a Split Marker Approach

25

Mark Arentshorst, Jing Niu, and Arthur F.J. Ram

25.1 Introduction

Targeted deletion of a Gene of Interest (GOI) is a powerful method to address gene functions and requires a double crossover homologous recombination (HR) event to exchange the GOI with a selection marker. In filamentous fungi, DNA integrates preferably via the nonhomologous end joining (NHEJ) pathway, which results in low frequencies of HR and consequently, in low efficiencies in obtaining gene deletion mutants. A successful approach to obtain gene deletion mutants with high efficiency has been the construction of mutants in the NHEJ-pathway, first described for *Neurospora crassa* (Ninomiya et al. 2004), and followed up by numerous other filamentous fungi including *Aspergillus niger* (Meyer et al. 2007; Carvalho et al. 2010; Arentshorst et al. 2012). Most often the fungal gene homologous to the gene encoding the Ku70 is used to generate a NHEJ mutant, but also Ku80 and Lig4 homologs have been disrupted to obtain NHEJ-deficient mutants (for reviews see Meyer 2008, Kuck and Hoff 2010 and references therein). The use of NHEJ mutants has greatly reduced time and effort to generate gene deletion mutants. The construction of a gene deletion cassette is also an important and time consuming factor. In principle, a gene deletion construct consists of a selection marker, flanked by upstream (5′) and downstream (3′) sequences of the GOI. Several approaches to generate gene deletion cassettes include traditional restriction enzyme and ligation-based cloning, GATEWAY cloning, fusion PCR, or in vivo assembly either in *Escherichia coli* or *Saccharomyces cerevisiae*.

An additional tool for improving gene targeting efficiencies is making use of the split marker technology. In this approach the gene deletion construct is split in two parts and each part contains the flanking region and a truncated form of the selection marker (Fairhead et al. 1996, Nielsen et al. 2006, Goswami 2012).

For the selection of transformants in *A. niger* (and also other filamentous fungi) the number of available markers is limited. Dominant selection markers for *A. niger* include markers giving resistance to hygromycin (pAN7.1) (Punt et al. 1987) or phleomycin (pAN8.1) (Punt and van den Hondel 1992), which are well established and commonly used. The uridine and arginine markers (*pyrG* (An12g03570) and *argB* (An14g03400), respectively), have been described earlier and are used in this study (Buxton et al. 1985; Van Hartingsveldt et al. 1987; Lenouvel et al. 2002). The *pyrG* gene encodes for the enzyme orotidine-5′-phosphate-decarboxylase and is required for uracil biosynthesis. The *argB* gene, encoding for an ornithine

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Table 25.1 Strains used in this study

Strain	Genotype	Description	Reference
N402	*cspA1*	derivative of N400	Bos et al. (1988)
AB4.1	*cspA1, pyrG378*	UV mutant of N402	Van Hartingsveldt et al. (1987)
MA169.4	*cspA1, pyrG378, kusA::DR-amdS-DR*	*ku70* deletion in AB4.1	Carvalho et al. (2010)
MA234.1	*cspA1, kusA::DR-amdS-DR*	*ku70* deletion in N402	Arentshorst (unpublished)
JN1.17	*cspA1, pyrG378, kusA::DR-amdS-DR, argB::hph*	*argB* deletion in MA169.4	Niu et al. (unpublished)
JN4.2	*cspA1, pyrG378, kusA::DR-amdS-DR, nicB::hph,*	*nicB* deletion in MA169.4	Niu et al. (unpublished)

carbamoyltransferase, is essential for arginine biosynthesis. In addition, a new auxotrophic mutant which requires nicotinamide for growth based on the *nicB* gene (An11g10910) was made. The *A. niger nicB* gene encodes a nicotinate–nucleotide pyrophosphorylase. Identification and the construction of a gene deletion cassette to disrupt *nicB* is based on a previous work by Verdoes et al. 1994, and will be described elsewhere in detail (Niu et al. manuscript in preparation). The *ΔnicB* strain is auxotrophic for nicotinamide and needs supplementation of nicotinamide to be able to grow. In addition, we reconstructed an *argB* deletion mutant (Niu et al. manuscript in preparation) to have all auxotrophic strains in the same strain background (Table 25.1).

Growth of all three auxotrophic strains (*pyrG*$^{-}$, *argB*$^{-}$, and *nicB*$^{-}$) on minimal medium requires the addition of uridine, L-arginine or nicotinamide, respectively,[1] and no growth is observed in the absence of the relevant supplements (data not shown). To minimize HR of the selection markers used in the disruption cassettes, the *argB* and *nicB* homologs from *Aspergillus nidulans* (ANID_04409.1 and ANID_03431.1 respectively) and the *pyrG* homolog from *Aspergillus oryzae* (AO090011000868) were PCR amplified. All genes are able to complement the auxotrophy of the relevant strain. The hygromycin and phleomycin cassettes also contain only nonhomologous sequences as both resistance genes are flanked by the *A. nidulans* *gpdA* promoter (*PgpdA*) and *trpC* terminator *(TtrpC)* (Table 25.2).

[1]The *argB* and *nicB* auxotrophic mutants are also *pyrG*$^{-}$ and therefore the growth medium for these strains needs to be supplemented with uridine.

25.2 General Methods

25.2.1 General Split Marker Approach

The split marker approach used for deleting the GOI is schematically depicted in Fig. 25.1 and consists of two overlapping DNA fragments to disrupt the GOI. The first fragment contains the 5′ flank of the GOI and a truncated version of the selection marker. The second DNA fragment contains an overlapping, but truncated version of the selection marker and the 3′ flank of the GOI. Both fragments are generated by fusion PCR as described below and transformed simultaneously to the recipient *A. niger* strain. The truncation of the selection marker at either site of the construct results in a nonfunctional marker and as a consequence transformation of only a single split marker fragment does not result in any transformants (data not shown).

25.2.2 Generation of Split Marker Fragments for *A. niger* Transformation

In this section the experimental design for creating the split marker fragments is discussed. The split marker DNA fragments can be obtained in three steps (Fig. 25.2). Each step is described in detail below.

Table 25.2 Plasmids to amplify selection markers

Plasmid	Selection marker	Remark	Reference
pAN7.1	Hygromycin; *hph*	*Pgpd* and *TtrpC* from *A.nidulans*	Punt et al. (1987)
pAN8.1	Phleomycin; BLE	*Pgpd* and *TtrpC* from *A. nidulans*	Punt and van den Hondel (1992)
pAO4-13	*pyrG*	*pyrG* from *A. oryzae*	De Ruiter-Jacobs et al. (1989)
pJN2.1	*argB*	*argB* from *A. nidulans*	Niu et al. (unpublished)
pJN4.1	*nicB*	*nicB* from *A. nidulans*	Niu et al. (unpublished)

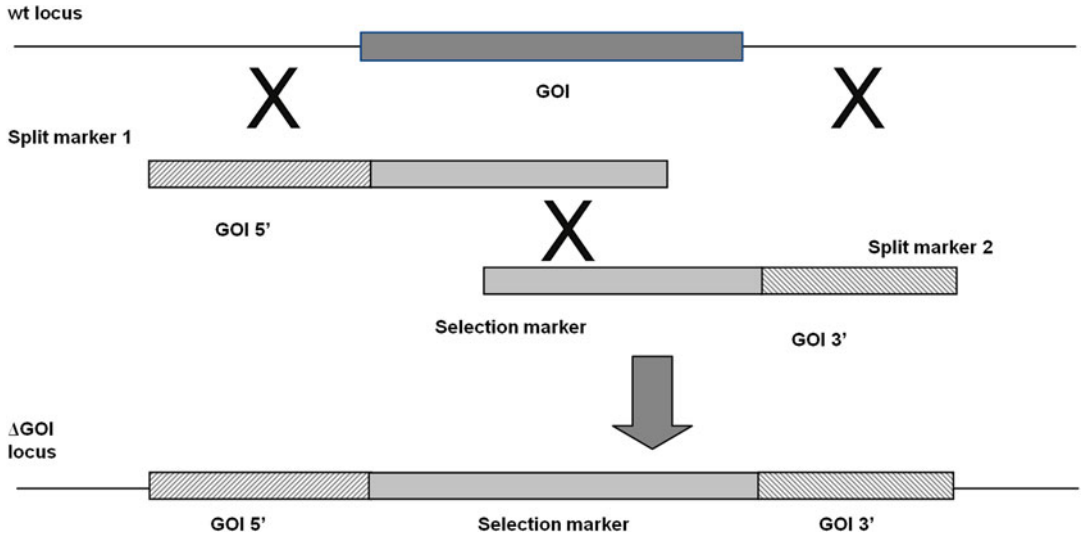

Fig. 25.1 Schematic representation of the split marker gene deletion approach. 5′ and 3′ sequences flanking the GOI (5′ and 3′) are transformed simultaneously to the recipient strain. By recombination of the selection maker and homologous integration of the cassette in the genome, a successful gene deletion mutant can be obtained

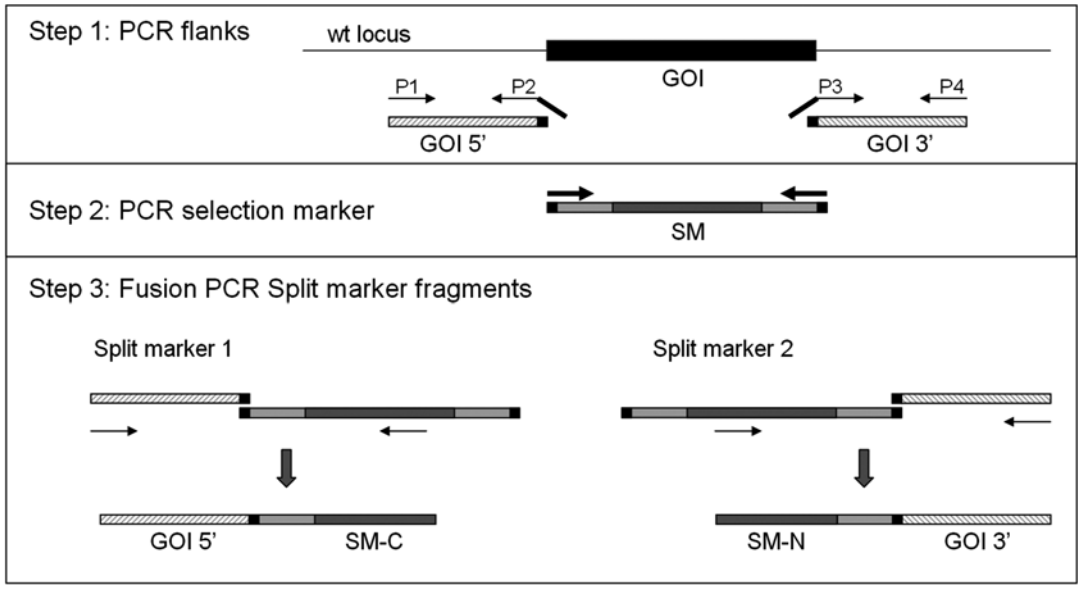

Fig. 25.2 Experimental design for creating split marker fragments

25.2.2.1 Experimental Design for Amplification of Flanking Regions of the GOI (Step 1)

Once the GOI has been identified, primers need to be designed for making gene deletion cassettes. First, two primers are required for the amplification of the 5′ flank of the GOI. The first primer (P1) is chosen between 700 and 900 bases upstream of the start codon. The reverse primer (P2) is as close to the start codon as possible and contains a 5′-CAATTCCAGCAGCGGCTT-3′ sequence, which is overlapping with all five selection markers and included for the subsequent fusion PCR. Also, two primers are required for the amplification of the 3′ flank of the GOI (P3 and P4). Again, the aim is to generate a 700–900 base pair long flank. In this case, the forward primer (P3) needs a 5′-ACACGGCACAATTATCCATCG-3′ sequence, which is also overlapping with all five selection markers for the subsequent fusion PCR (Step 3).

25.2.2.2 Experimental Design for Amplification of Suitable Selection Marker (Step 2)

For the amplification of the PCR fragments containing the appropriate selection marker the following plasmids can be used (see also Table 25.2):

The plasmid pAN7.1 (Punt et al. 1987) is used as template to amplify the hygromycin resistance cassette, containing the *hph* gene from *E. coli*, coding for hygromycin B phosphotransferase. Expression of the *hph* gene is driven by the *A. nidulans gpdA* promoter, and terminated by the *A. nidulans trpC* terminator. The plasmid pAN8.1 (Punt and van den Hondel 1992) is used as template to amplify the phleomycin resistance cassette, containing the BLE gene from *Streptoalloteichus hindustanus*, coding for a phleomycin-binding protein. Expression of the BLE gene is also driven by the *A. nidulans gpdA* promoter and terminated by the *A. nidulans trpC* terminator. The plasmid pAO4-13 (De Ruiter-Jacobs et al. 1989) is used as template to amplify the *A. oryzae pyrG* gene (AO090011000868), including promoter and terminator region. The *argB* gene (ANID_04409.1) and the *nicB* gene (ANID_03431.1) of *A. nidulans*, including promoter and terminator region, were amplified using primer pairs argBnidP5f and argBnidP6r or nicBnidP5f and nicBnidP6r, and genomic DNA of *A. nidulans* strain FGSC A234 (*yA2, pabaA1, veA1*), obtained from the Fungal Genetics Stock Center, as template. The resulting PCR products were ligated into PCR-cloning vector pJet1.2 (K1231, Thermo Fisher), to give plasmids pJN2.1 and pJN4.1 respectively (Table 25.2). Plasmid pJN2.1 and pJN4.1 can be used to amplify the *argB* gene or the *nicB* gene.

We developed a generic split marker approach in such a way that with a single set of four GOI primers, all five different selection markers can be used to generate the deletion cassette. Each primer, used to amplify a specific selection marker (Fig. 25.3, Table 25.3), contains sequences which are overlapping with the GOI primer sequences (see Sect. 25.2.2.1) to create gene deletion mutants with either one of the different selection markers.

25.2.2.3 Experimental Design for the Generation of Split Marker Fragments (Step 3)

Once both flanks of the GOI (Fig. 25.2, step 1) and the required selection marker (Fig. 25.2, step 2 and Fig. 25.3) have been amplified, the split marker fragments can be obtained by fusion PCR (Fig. 25.2, step 3). Exact details are described in Sect. 25.3.2.3. After column purification, the resulting split marker fragments can directly be used to transform *A. niger*.[2]

25.3 Detailed Procedure Description

As proof of principle, the *A. niger amyR* gene (An04g06910), encoding the amylase transcriptional regulator, has been used. The *ΔamyR* strain cannot grow on starch, allowing an easy screen for *ΔamyR* transformants (Petersen et al. 1999). This section contains a detailed description of the whole procedure of deleting *amyR*, using all five

[2] A small sample of PCR fragments is routinely analyzed for purity and size. Optional is to confirm PCR product integrity by restriction analysis or sequencing.

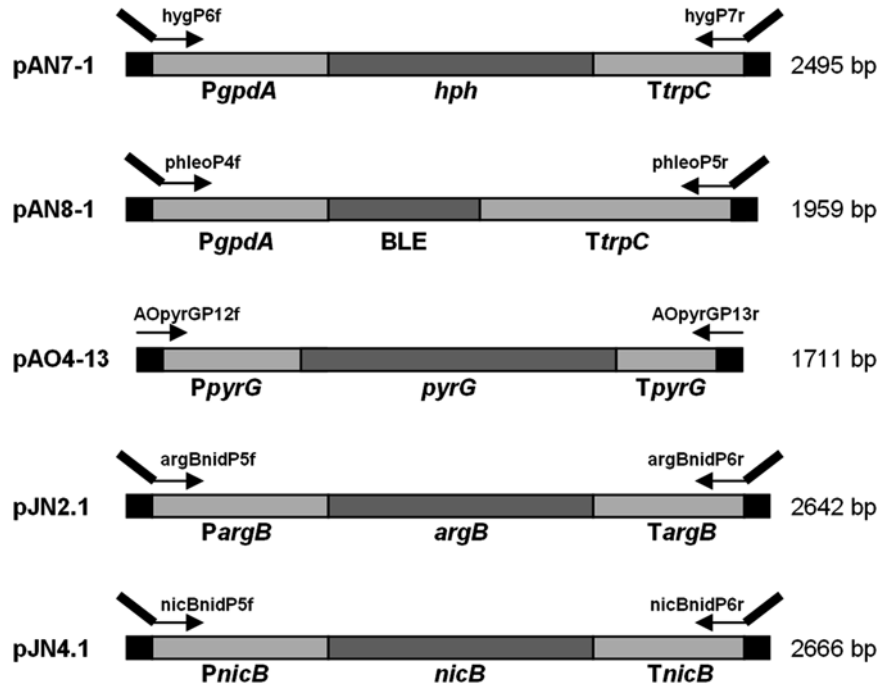

Fig. 25.3 PCR products for all five selection markers. Overlapping sequences of the primers are indicated by bold lines. The size of the PCR products is indicated for each selection marker

Table 25.3 Primers used to generate selection markers

Primer name	Sequence (5′–3′)	Remark	Template
hygP6for	**AAGCCGCTGCTGGAATTG-**GGCTCTGAGGTGCAGTGGAT	Amplification of hph marker	pAN7.1
hygP7rev	**CGATGGATAATTGTGCCGTGT-**TGGGTGTTACGGAGCATTCA	Amplification of hph marker	pAN7.1
phleoP4for	**AAGCCGCTGCTGGAATTG - **CTCTTTCTGGCATGCGGAG	Amplification of BLE marker	pAN8.1
phleoP5rev	**CGATGGATAATTGTGCCGTGT-**GGAGCATTCACTAGGCAACCA	Amplification of BLE marker	pAN8.1
AOpyrGP12f	**AAGCCGCTGCTGGAATTG**	Amplification of *pyrG* marker	pAO4-13
AOpyrGP13r	**CGATGGATAATTGTGCCGTGT**	Amplification of *pyrG* marker	pAO4-13
argBnidP5f	**AAGCCGCTGCTGGAATTG - **TTTCGACCTCTTTCCCAATCC	Amplification of *argB* marker	pJN2.1
argBnidP6r	**CGATGGATAATTGTGCCGTGT-**TCCTGTGGGTCTTTGTCCG	Amplification of *argB* marker	pJN2.1
nicBnidP5f	**AAGCCGCTGCTGGAATTG-**CGTTATGCACAGCTCCGTCTT	Amplification of *nicB* marker	pJN4.1
nicBnidP6r	**CGATGGATAATTGTGCCGTGT-**GCGCATACACAGAAGCATTGA	Amplification of *nicB* marker	pJN4.1

Note: Overlapping sequences for fusion PCR are indicated in *bold*

Table 25.4 GOI (amyR) specific primers to amplify 5′ and 3′ flanks

Primer name	Sequence (5′–3′)	Remark
amyRP7f	ATCGTCAGCGAGCCTCAGA	Amplification of *amyR* 5′ flank
amyRP8r	**CAATTCCAGCAGCGGCTT-**TTGTATGCGGAGACAAGTGTGAC	Amplification of *amyR* 5′ flank
amyRP9f	**ACACGGCACAATTATCCATCG-**CCCTCATGAACAAGAAGCAGC	Amplification of *amyR* 3′ flank
amyRP10r	GAGGACGCCATCATTGACG	Amplification of *amyR* 3′ flank

Note: Overlapping sequences for fusion PCR are indicated in *bold*

Table 25.5 Generic primers used to amplify bipartite fragments

Primer name	Sequence (5′–3′)	Remark
hygP9r	GGCGTCGGTTTCCACTATC	Reverse primer split marker fragment 1 *hph*
hygP8f	AAAGTTCGACAGCGTCTCC	Forward primer split marker fragment 2 *hph*
phleoP7r	CACGAAGTGCACGCAGTTG	Reverse primer split marker fragment 1 BLE
phleoP6f	AAGTTGACCAGTGCCGTTCC	Forward primer split marker fragment 2 BLE
AOpyrGP15r	CCGGTAGCCAAAGATCCCTT	Reverse primer split marker fragment 1 *pyrG*
AOpyrGP14f	ATTGACCTACAGCGCACGC	Forward primer split marker fragment 2 *pyrG*
argBnidP8r	TGGTTTGCAGAAGCTTTCCTG	Reverse primer split marker fragment 1 *argB*
argBnidP7f	ACTCCTCGCAAACCATGCC	Forward primer split marker fragment 2 *argB*
nicBnidP8r	GAACAGCCTTCGGGATTGC	Reverse primer split marker fragment 1 *nicB*
nicBnidP7f	CGCCTTATATCCGATTGGCTT	Forward primer split marker fragment 2 *nicB*

selection markers, illustrated with results of the experiments. Sequences of all primers used are listed in Tables 25.3, 25.4, and 25.5.

25.3.1 Materials and Reagents

For the medium composition of minimal medium, the preparation of stock solutions for the medium and for a detailed protocol of genomic DNA isolation of *A. niger* we refer to the Materials and Reagents section in Arentshorst et al. 2012.

1. PCR enzyme (we routinely use Phire Hot start II DNA Polymerase [F-122 L, Thermo Fisher]).
2. dNTPs (1.25 mM): Add 0.25 mL of all 4 dNTPs (dNTP Set 100 mM Solutions (4×0.25 mL, R0181, Thermo Fisher)) to 19 mL of MQ, mix well, make aliquots of 0.5 mL, and store at −20 °C.
3. PCR purification Kit (we routinely use Genejet Gel Extraction Kit (K0692, Thermo Fisher), also for PCR purifications).
4. Hygromycin (100 mg/mL): Dissolve 1 g of hygromycin (InvivoGen, ant-hg-10p) in 10 mL of MQ, sterilize by filtration, make aliquots of 500 µL, and store at −20 °C. The final concentration in the medium is 100 µg/mL, except for transformation plates, then use 200 µg/mL.
5. Phleomycin (40 mg/mL), for 10 mL: add 400 mg of phleomycin (InvivoGen, ant-ph-10p) to 8 mL of warm MQ (~60 °C) in a 15 mL tube. When phleomycin is dissolved, add MQ up to 10 mL, and filter sterilize. Make aliquots and store at −20 °C.
6. Uridine (1 M), for 100 mL: add 22.4 g of uridine (Acros, 140775000) to 50 mL of warm MQ (~60 °C) in a 100 mL cylinder. When uridine is dissolved, add MQ up to 100 mL, sterilize by filtration, and store at 4 °C. Final concentration in medium is 10 mM.
7. Arginine (2 %), for 100 mL: add 2 g of L-arginine monohydrochloride (Sigma, A5131) to 50 mL of warm MQ (~60 °C) in a 100 mL cylinder. When arginine is dissolved,

add MQ up to 100 mL, sterilize by filtration, and store at 4 °C.

8. Nicotinamide (0.5 %), for 100 mL: add 0.5 g of nicotinamide (Sigma, N0636) to 50 mL of warm MQ (~60 °C) in a 100 mL cylinder. When nicotinamide is dissolved, add MQ up to 100 mL, sterilize by filtration, and store at 4 °C.
9. Transformation media + phleomycin: Prepare MMS and Top agar according to Arentshorst et al. 2012. After autoclaving, and cooling down to 50 °C, add phleomycin to a final concentration of 50 μg/mL, to both the MMS and the Top agar.
10. MM + agar + L-arginine: Prepare 500 mL of MM + agar according to Arentshorst et al. 2012. Add 5 mL of 2 % L-arginine after autoclaving (100× dilution).
11. MM + agar + nicotinamide: Prepare 500 mL of MM + agar according to Arentshorst et al. 2012. Add 0.25 mL of 0.5 % nicotinamide after autoclaving (2,000× dilution).
12. MM + agar + starch: For 500 mL: Dissolve 5 g of starch (soluble, extra pure, Merck, 1.01253) in 450 mL of warm MQ (~60 °C). Add 10 mL of 50× ASP + N, 1 ml of 1 M $MgSO_4$, 50 μL of trace element solution, 15 mg of yeast extract (YE)[3] (Roth, 2363.2) and 7.5 g agar bact. (Scharlau, 07-004-500), and autoclave.

25.3.2 Methods

25.3.2.1 Amplification of the AmyR 5′- and 3′ Flank

1. *AmyR* primers were designed (Fig. 25.2, Step 1 and Table 25.4), and subsequently used in PCR reactions to amplify both the *amyR* 5′ flank and 3′ flank.
2. The PCR mix, total volume of 50 μL, contained 1 μL genomic DNA of *A. niger* wt strain N402 (1 μg/μL), 8 μL dNTPs (1.25 mM), 10 μL 5× Phire buffer, 1 μL Primer F (20 pmol/μL), 1 μL Primer R (20 pmol/μL), 0.5 μL Phire Hot start II DNA Polymerase and 28.5 μL of MQ.
3. PCR was performed under the following conditions: initial denaturation for 5 min at 98 °C, 30 cycles of 5 s at 98 °C, 5 s at 58 °C, and 15 s per 1 kb of template at 72 °C, followed by final extension of 5 min at 72 °C.
4. PCR reactions were analyzed by loading 5 μL PCR reaction on a 1 % agarose gel.
5. After column purification and elution with 30 μL of MQ, DNA concentration for both flanks was ~37 ng/μL.

25.3.2.2 Amplification of the Selection Markers

1. Primers for all five selection markers were designed (Fig 25.3, Table 25.3) and used for PCR. In these PCR reactions 1 ng of plasmid (pAO4-13, pAN7.1, pAN8.1, pJN2.1, and pJN4.1, respectively) was used as template. For PCR mix and PCR conditions see Sect. 25.3.2.1.
2. After confirmation on agarose gel, selection marker PCR products were column purified, yielding DNA concentrations of ~50 ng/μL. The markers were stored at −20 °C and used repeatedly.

25.3.2.3 Amplification of the Split Marker Fragments

1. Fusion PCR fragments were amplified according to Fig. 25.2, step 3 (see also Tables 25.5 and 25.6). Both *amyR* flanks and all selection markers (Sects. 25.3.2.1 and 25.3.2.2) were diluted to 2 ng/μL.
2. For each PCR reaction, 2 ng of *amyR* flank and 2 ng of selection marker PCR were used as template (Table 25.6). For PCR mix and PCR conditions see Sect. 25.3.2.1.
3. Two identical fusion PCR reactions were performed, in order to increase the yield of PCR product.
4. Fusion PCR products were analyzed on agarose gel, followed by column purification. The DNA concentration for all fragments

[3]YE is added to a final concentration of 0.003 % to stimulate germination of *A. niger*. On MM + starch without YE, the wt strain also does not germinate very well.

Table 25.6 Overview of templates and primers used in Fusion PCR reactions to obtain split marker fragments

Split Marker 1					Split Marker 2					Transformed
Template		Primer		Product	Template		Primer		Product	Strain
1	2	Forward	Reverse	Yield (μg)	1	2	Forward	Reverse	Yield (μg)	
5′amyR	PCR hph	amyRP7f	hygP9r	2.5	3′amyR	PCR hph	hygP8f	amyRP10r	2.6	MA234.1
5′amyR	PCR BLE	amyRP7f	phleoP7r	2.6	3′amyR	PCR BLE	phleoP6f	amyRP10r	2.6	MA234.1
5′amyR	PCR pyrG	amyRP7f	AOpyrG15r	2.8	3′amyR	PCR pyrG	AOpyrGP14f	amyRP10r	2.8	MA169.4
5′amyR	PCR argB	amyRP7f	argBnidP8r	3.0	3′amyR	PCR argB	argBnidP7f	amyRP10r	3.2	JN1.17
5′amyR	PCR nicB	amyRP7f	nicBnidP8r	3.3	3′amyR	PCR nicB	nicBnidP7f	amyRP10r	2.5	JN4.2

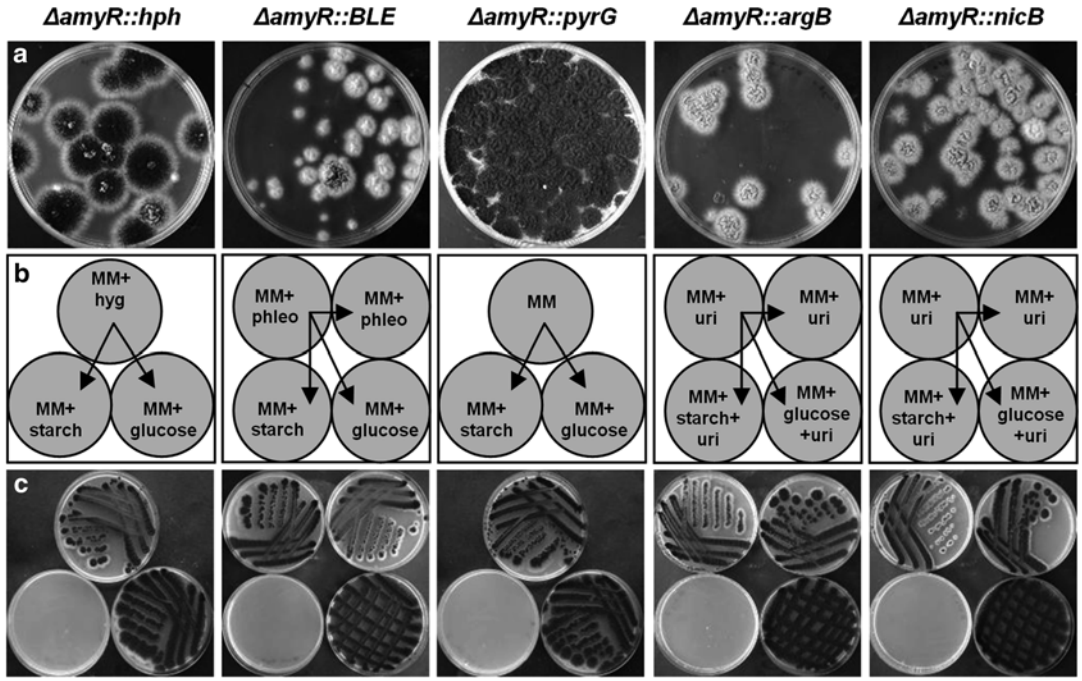

Fig. 25.4 Phenotypic analysis of putative *amyR* disruptant strains using five different selection markers (*hph*, hygromycin resistance; BLE, phleomycin resistance; *pyrG*, uridine requiring; *argB*, arginine requiring; *nicB*, nicotinamide requiring). (**a**) Transformation plates after transforming split marker fragment combinations for each of the five *amyR* deletion cassettes to the relevant recipient strain (Table 25.6). (**b**, **c**) Purified transformants were analyzed for their ability to grow on starch. The inability to grow on starch is indicative for the deletion of the *amyR* gene

varied between 120 and 160 ng/μL in a total volume of 20 μL (Table 25.6, column DNA Yield).[4]

25.3.2.4 Transformation of Split Marker Fragments to *A. niger* Δku70 Strains

1. Split marker fragments were combined and transformed to different *A. niger* strains (Table 25.6, column Transformed strain), according to Arentshorst et al. 2012. Results of these transformations are shown in Fig. 25.4.
2. As a control, also separate split marker fragments were transformed. None of the separately transformed split marker fragments yielded any transformants (data not shown).
3. Four transformants were purified for each selection marker tested.[5] For purification protocol, see Arentshorst et al. 2012.
4. After the second purification, all purified transformants were tested for growth on MM + starch (Fig. 25.4). All transformants analyzed showed a *ΔamyR* phenotype.
5. Purified transformants can be further analyzed by isolating genomic DNA, followed by both Southern blot analysis and diagnostic PCR (Arentshorst et al. 2012).

[4] The split marker fragments are not purified from gel and template DNA (*pyrG, hygB, Ble*, *argB*, and *nicB* genes, respectively) used for amplification of the split marker might remain present in the next steps. We therefore include control transformations with both split markers separately. As no transformants are obtained in the transformation with only one flank (data not shown), the purification of the split marker fragment is not required, but is optional.

[5] Only the sporulating transformants on the phleomycin transformation plate (see Fig. 25.4) can grow on MM + phleomycin. The non-sporulating transformants do not grow, and are probably transient transformants, in which the split marker fragments have not integrated into the genome.

25.3.2.5 Transformation of Split Marker Fragments to *A. niger* wt Strains

For some experimental set-ups, it is preferred to analyze gene deletions in a *ku70* wild-type strain. In order to show that the split marker approach also can be applied to a wild-type (*ku70* plus) strain, both *A. niger* strains AB4.1 (Van Hartingsveldt et al. 1987) (*pyrG*$^{-}$) and MA169.4 (*Δku70, pyrG*$^{-}$) were transformed with *ΔamyR::pyrG* split marker fragments. After purification and screening on MM + starch, 25 out of 60 AB4.1-transformants (41 %) showed a *ΔamyR* phenotype.[6] For MA169.4, 39 out of 40 transformants (98 %) showed a *ΔamyR* phenotype. This result clearly shows that the split marker approach can also be used to make gene deletions in a wt background instead of a *Δku70* background.

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[6] The percentages of HR for the *amyR* gene are very high (41 % for wt, 98 % for Δ*ku70*). Usually we find 5–10 % HR for wt, and 80–100 % for Δ*ku70* (Meyer et al. 2007).

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REMI in Molecular Fungal Biology

26

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26.1 Introduction

The principle of restriction enzyme mediated integration (REMI) is straightforward. Restriction enzyme is added during PEG transformation of protoplasts or during electroporation of fungal cells or conidia. Usually, the transforming DNA is linearized with the same enzyme as added to the transformation mixture. REMI was first applied to investigate illegitimate recombination in *Saccharomyces cerevisiae* (Schiestl and Petes 1991). The primary use of REMI, however, has been gene tagging by insertional mutagenesis and, as such, was first used in the soil amoeba *Dictyostelium discoideum* (Kuspa and Loomis 1992). REMI has been used successfully for this reason in a wide variety of ascomycetes and basidiomycetes (Table 26.1). This technique revealed genes involved in fungal pathogenicity, the production of mycotoxins and secondary metabolites, heterologous protein expression, drug resistance, development, and cellular processes (Bölker et al. 1995; Sweigard et al. 1998; Balhadère et al. 1999; Thon et al. 2000; Yaver et al. 2000; Kimura et al. 2001; Yakoby et al. 2001; Chung et al. 2002; Fujimoto et al. 2002; Busch et al. 2003; Chen et al. 2005; Seong et al. 2005; Shim et al. 2006; Shim and Woloshuk 2001; De Souza et al. 2000; Bowyer et al. 2012; Choi et al. 2008; Cummings et al. 1999; Makino and Kamada 2004; Muraguchi et al. 2008; Nakazawa et al. 2010; Stromhaug et al. 2001; Mukaiyama et al. 2002; Larsen et al. 2013). *Mucor circinelloides* is the only zygomycete in which REMI has been used (Papp et al. 2013). In this case REMI was compared to normal PEG and *Agrobacterium tumefaciens* mediated transformation (AMT) for enhanced production of canthaxanthin. REMI and normal PEG mediated transformation but not AMT resulted in stable transformants.

The addition of restriction enzyme during protoplast transformation generally increases transformation efficiency, results in more single locus integrations, and can make integration events more random. This has made REMI a valuable technique for functional genomics in fungi. Here we describe the mechanism of vector integration during REMI, its historical use, and its advantages and disadvantages. We will also discuss the role of REMI in fungal molecular biology in the era of next-generation sequencing.

26.2 The Mechanism of REMI

Restriction enzyme added to the transformation mixture is thought to cleave genomic DNA of recipient cells. This causes double stranded breaks (DSB), which activates the DNA damage

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Table 26.1 Organisms used for REMI transformations

Phylum	Organism	Publication	Success
Ascomycota	*A. alternata*	Akamatsu et al. (1997)	++
		Tanaka et al. (1999)	
	Arthrobotrys oligospora	Tunlid et al. (1999)	+
		Jin et al. (2005b)	
	Aspergillus fumigatus	Brown et al. (1998)	++
		Bowyer et al. (2012)	
	Aspergillus nidulans	Sanchez et al. (1998)	++
		De Souza et al. (2000)	
		Busch et al. (2003)	
		Soid-Raggi et al. (2006)	
	A. niger	Shuster and Connelley (1999)	+
	Aspergillus oryzae	Yaver et al. (2000)	+
	Beauveria bassiana	Jiang et al. (2007)	±
	Bipolaris eleusines	Jianping et al. (2012)	+
	C. albicans	Brown et al. (1996)	++
		Brown et al. (1999)	
		Chen and Kumamoto (2006)	
	C. famata	Dmytruk et al. (2006)	−
	C. nicotianae	Chung et al. (2003)	±
	C. heterostrophus	Lu et al. (1994)	+
	C. acutatum	Chung et al. (2002)	−
		Chen et al. (2005)	
		You et al. (2007) (AMT)	
		Talhinhas et al. (2008) (AMT)	
	Colletotrichum gloeosporioides	Yakoby et al. (2001)	+
		Horowitz et al. (2004)	
	C. graminicola	Epstein et al. (1998)	+
		Thon et al. (2000)	
	Colletotrichum lagenarium	Kimura et al. (2001)	+
		Takano et al. (2006)	
	Colletotrichum lindemuthianum	Redman and Rodriguez (1994)	±
	C. magna	Redman et al. (1999)	++
	C. minitans	Rogers et al. (2004)	−
	Curvularia lunata	Wang et al. (2013)	+
	F. graminearum (*Gibberella zeae*)	Han et al. (2004)	++
		Seong et al. (2005)	
		Seong et al. (2006)	
		Kim et al. (2007)	
		Ramamoorthy et al. (2007)	
	F. oxysporum	Inoue et al. (2001)	++
		Namiki et al. (2001)	
		Kawabe et al. (2004)	
		Morita et al. (2005)	
		Yoshida et al. (2008)	
	Fusarium verticillioides	Shim and Woloshuk (2001)	++
		Shim et al. (2006)	
		Choi et al. (2008)	
		Ridenour et al. (2013)	

(continued)

Table 26.1 (continued)

Phylum	Organism	Publication	Success
	G. fujikuroi	Linnemannstons et al. (1999)	±
	H. polymorpha	Van Dijk et al. (2001)	−
	L. maculans	Sexton and Howlett (2001)	±
		Idnurm and Howlett (2002)	
		Idnurm and Howlett (2003)	
	M. grisea	Shi et al. (1995)	−
		Sweigard et al. (1998)	
		Balhadère et al. (1999)	
		Rho et al. (2001)	
		Fujimoto et al. (2002) (AMT)	
		Jeon et al. (2007) (AMT)	
	Monacrosporium sphaeroides	Jin et al. (2005a)	+
	Mycosphaerella zeae-maydis	Yun et al. (1998)	±
	Ozonium (endophytic fungus BT2)	Wang et al. (2007)	±
	Paecilomyces lilacinus	Yang et al. (2011)	±
	P. paxilli	Itoh and Scott (1997)	+
		Young et al. (2001)	
	Phomopsis viticola	De Guido et al. (2003)	±
	Pichia pastoris	Stromhaug et al. (2001)	++
		Mukaiyama et al. (2002)	
		Farre et al. (2007)	
		Larsen et al. (2013)	
	Pichia stipitis	Cho and Jeffries (1998)	−
		Maassen et al. (2008)	
	S. cerevisiae	Schiestl and Petes (1991)	++
		Schiestl et al. (1994)	
		Manivasakam and Schiestl (1998)	
		Chan et al. (2007)	
	Trichoderma atroviride	Tang et al. (2009)	±
	Trichoderma koningii	Zhou et al. (2007)	±
		Wang et al. (2009)	
	T. mentagrophytes	Kaufman et al. (2004)	±
Basidiomycota	*C. cinereus*	Granado et al. (1997)	++
		Cummings et al. (1999)	
		Inada et al. (2001)	
		Makino and Kamada (2004)	
		Muraguchi et al. (2008)	
		Nakazawa et al. (2010)	
	Coprinus congregatus	Leem et al. (1999)	±
		Leem et al. (2003)	
	Flammulina velutipes	Maehara et al. (2010)	+
	Ganoderma lucidum	Kim et al. (2004)	±
	H. cylindrosporum	Combier et al. (2004)	−
	L. edodes	Sato et al. (1998)	+
		Hirano et al. (2000)	
		Sato et al. (2011)	
	Moniliophthora perniciosa	Lopes et al. (2008)	+

(continued)

Table 26.1 (continued)

Phylum	Organism	Publication	Success
	Pleurotus eryngii	Noh et al. (2010)	+
	Pleurotus ostreatus	Irie et al. (2001)	+
	U. maydis	Bölker et al. (1995)	+
		Aichinger et al. (2003)	
	Trametes versicolor	Kim et al. (2002)	±
	Tremella fuciformis	Zhu et al. (2006)	±
Zygomycota	*M. circinelloides*	Papp et al. (2013)	±
Amoebozoa	*D. discoideum*	Kuspa and Loomis (1992)	++
		Kuspa and Loomis (1994)	
		Hsu et al. (2011)	
		Couto et al. (2011)	
	Polysphondylium	Fey and Cox (1997)	±

response (DDR) (Ciccia and Elledge 2010; Deriano and Roth 2013). Activation of DDR results in DSB repair by homologous recombination or by nonhomologous end joining (NHEJ). It is generally accepted that the NHEJ pathway is primarily responsible for vector integration during REMI (Riggle and Kumamoto 1998; Hsu et al. 2011; Couto et al. 2011). This is based on several observations and experimental evidence. For instance, ectopic integrations mediated by NHEJ occur more frequent in fungi than homologous integration (Fincham 1989). Furthermore, it was shown in *S. cerevisiae* that REMI occurs independently of the homologous recombination pathway (which is dominant in this yeast, Schiestl et al. 1994), while inactivation of a component of the NHEJ pathway dramatically reduced REMI in *D. discoideum* (Hsu et al. 2011; Couto et al. 2011).

Two variants of NHEJ are distinguished, namely classical and alternative NHEJ. Classical NHEJ (cNHEJ) requires the proteins Ku, DNA-PKcs, Artemis, XRCC4, ligase IV, and Cernunnos/XLF. Alternative NHEJ (aNHEJ) is thought to act independently of Ku and DNA-PKcs, and microhomology (i.e., short homologous regions of up to 10 nucleotides) is more important than in cNHEJ (Fig. 26.1, McVey and Lee 2008; Deriano and Roth 2013). Moreover, aNHEJ results in the loss of DNA from its ends and chromosomal translocations can occur (Deriano and Roth 2013). Members of the poly(ADP-ribose) polymerases (PARP) family can be involved in both cNHEJ and aNHEJ. For example, PARP1 is involved in the aNHEJ pathway of mammalian cells while PARP3 is involved in cNHEJ (Pears et al. 2012). Inactivation of *ku70*, *ku80*, the Artemis homologue *dclre1*, or the PARP family member *adprt1a* dramatically reduces REMI in *D. discoideum* (Hsu et al. 2011; Couto et al. 2011). This indicates that the cNHEJ pathway is responsible for REMI in this microbe. In *S. cerevisiae*, REMI results in microhomology-mediated recombination (MHMR, Manivasakam and Schiestl 1998; Chan et al. 2007). This suggests the involvement of aNHEJ. However, DNA ends are protected from degradation, implying involvement of the cNHEJ pathway (Deriano and Roth 2013). Thus, it is not yet clear which mechanism operates in *S. cerevisiae.* It is clear that the NHEJ pathway in *S. cerevisiae* can generate integrations *in trans* of a cleaved restriction site in the genome. This was concluded from the finding that expression of the endonuclease I-SceI in a *S. cerevisiae* strain that contained a unique I-SceI recognition site resulted in MHMR throughout its genome (Chan et al. 2007). Inactivation of *ku70* or *ku80* has resulted in a relatively higher frequency of homologous recombination in several fungi (Krappmann 2007; De Jong et al. 2010; Salame et al. 2012; Nakazawa et al. 2011). Basically, inactivation of these genes abolishes the cNHEJ pathway (Fig. 26.1). Therefore, the cNHEJ pathway is likely the dominant NHEJ pathway in these fungi.

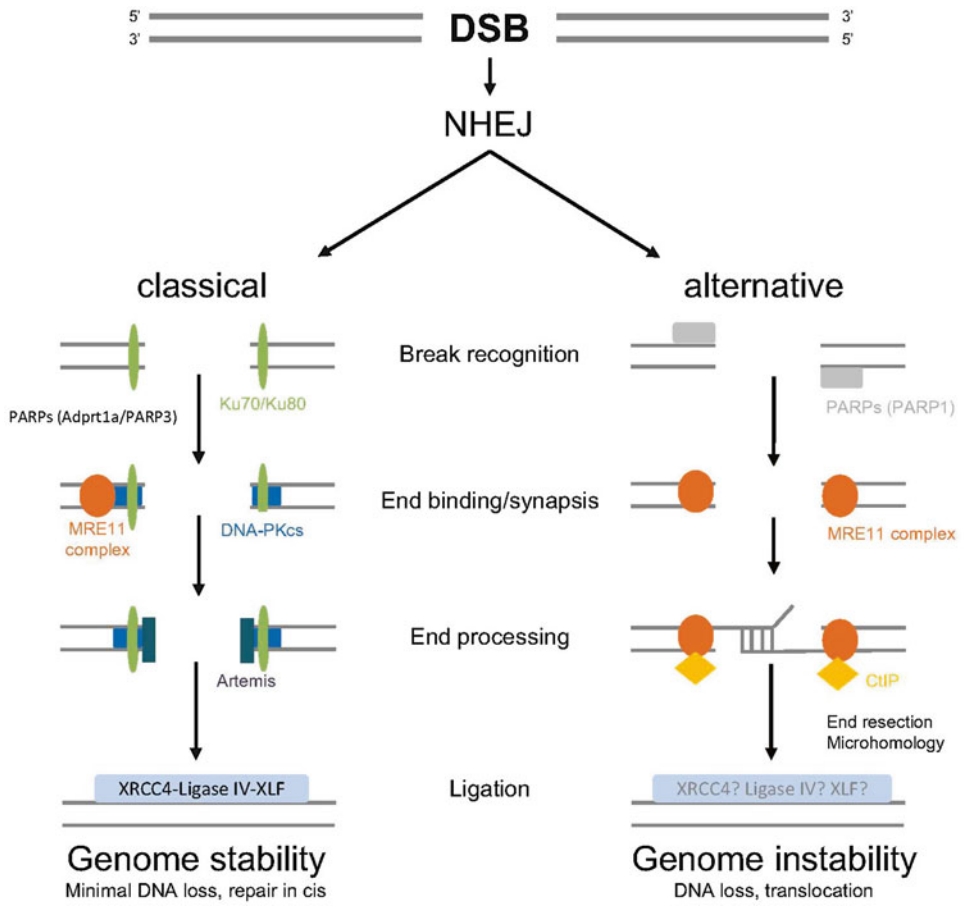

Fig. 26.1 Model of cNHEJ and aNHEJ. (Adapted from Deriano, L. & Roth, D.B. 2013, "Modernizing the Nonhomologous End-Joining Repertoire: Alternative and Classical NHEJ Share the Stage", Annual Review of Genetics, vol. 47, pp. 433–455 with permission)

Double stranded DNA breaks can be repaired by simple ligation of compatible ends or by processed recombination (Fig. 26.2, Daley et al. 2005). Genomic integrations are considered true REMI events if the restriction sites bordering the integrated linearized vector are restored. This corresponds to the simple ligation repair of DSB. True REMI events are identified through Southern blot analysis of genomic DNA digested with the enzyme used for REMI. Hybridizing fragments with the same size as the linearized vector are indicative of true REMI events. Processed recombination of vector DNA destroys the restriction sites bordering the linear DNA fragment. Therefore, integration events which do not result in restoration of the flanking restriction sites can still be the result of REMI. In REMI, the same restriction enzyme used to linearize the transforming DNA is usually used for co-transformation. This was shown to be beneficial or even essential for efficient REMI in some cases (Kuspa and Loomis 1992; Shi et al. 1995; Manivasakam and Schiestl 1998; Thon et al. 2000). However, linearized plasmid is not always required (Granado et al. 1997).

Efficiency of REMI integrations varies in fungi. For example, true REMI events occur with a frequency of 100 %, 68–80 %, 72 %, 50 %, 6 %, and 4 %, in *Candida albicans*, *S. cerevisiae*, *Magnaporthe grisea*, *Lentinus edodes*, *Alternaria alternata*, and *Gibberella fujikuroi*, respectively (Brown et al. 1996; Schiestl and Petes 1991;

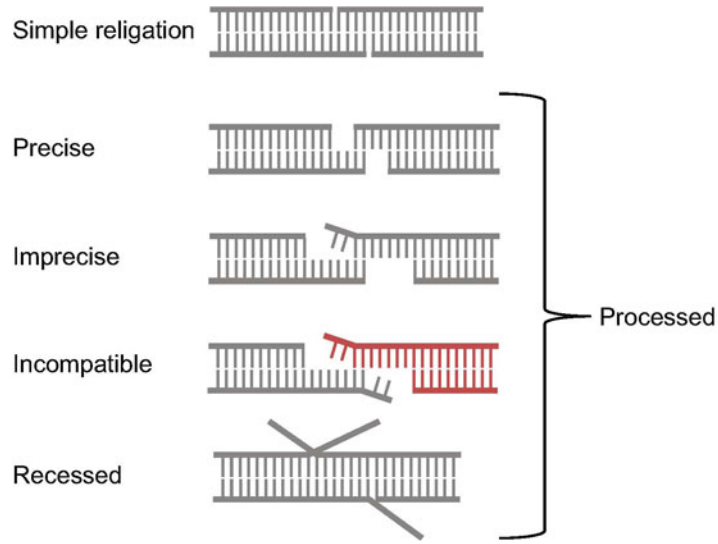

Fig. 26.2 Simple and processed double stranded DNA repair in *S. cerevisiae*. (Adapted from Daley, J.M., Palmbos, P.L., Wu, D. & Wilson, T.E. 2005, "Nonhomologous end joining in yeast", Annual Review of Genetics, vol. 39, pp. 431–451 with permission)

Manivasakam and Schiestl 1998; Shi et al. 1995; Sato et al. 1998; Hirano et al. 2000; Akamatsu et al. 1997; Linnemannstons et al. 1999). The type of restriction enzyme used for REMI may also impact frequency of true REMI events. For example, this frequency was 72 % when *Bam*HI was used for REMI in *M. grisea*, while *Hind*III and *Eco*RV resulted in a frequency of 28 % and 42 %, respectively (Shi et al. 1995). The effect of restriction enzymes on true REMI events in a fungal species cannot be predicted. This may be caused by unknown species specific properties of the DNA repair machinery.

The addition of restriction enzymes during a fungal transformation may result in increased transformation efficiency. This increase in efficiency is influenced by the type and concentration of restriction enzyme. REMI in *Colletotrichum graminicola*, *Coprinus cinereus*, *L. edodes*, *Colletotrichum magna*, *Hansenula polymorpha*, *Candida famata*, and *Fusarium oxysporum* increased transformation efficiency up to 27, 7, 2–4, 2, 1.8, 1.8, and 1-fold, respectively (Thon et al. 2000; Granado et al. 1997; Sato et al. 1998; Redman et al. 1999; Van Dijk et al. 2001; Dmytruk et al. 2006; Inoue et al. 2001). Similarly, REMI with *Bam*HI and *Bgl*II generated more transformants in *M. grisea*, than *Hind*III and *Eco*RV (Shi et al. 1995). Of the seven restriction enzymes tested in *C. magna* (*Xba*I, *Hind*III, *Sac*I, *Sal*I, *Nde*I, ApaI, *Pvu*II) *Hind*III performed best (Redman et al. 1999). The optimal restriction enzyme concentration usually depends on the enzyme used. For example, the optimal concentration of *Bam*HI, *Eco*RI, and *Pst*I in *C. cinereus* was 20–40, 40–60, and 80–100 units, respectively (Granado et al. 1997). This resulted in 300, 800, and 400 transformants per µg of vector DNA. Differences in effectiveness may be caused by the efficiency of the transfer of the enzyme to the nucleus or its stability in the external and internal environment of the cell. Increased transformation efficiency is generally higher when the enzyme added during REMI produces compatible ends to the linearized vector (Manivasakam and Schiestl 1998). Note that REMI can also result in lower transformation efficiency; a reduction of 10-fold in *Cercospora nicotianae* was observed when REMI was used (Chung et al. 2003).

Genomic integration of plasmid DNA in REMI can result in more random genomic integration sites. In *G. fujikuroi*, 6.5 % of non-REMI transformants were gibberellin deficient. On the other hand, this was only 0.1 % in REMI transformants. In all cases, the phenotype was the result of a deletion which was attributed to an integration event in or near genes involved in

gibberellin synthesis (Linnemannstons et al. 1999). This illustrates a reduction in hotspot integration due to REMI. It is unclear what drives this effect. It should be noted that REMI will never be truly random since restriction enzyme sites are not evenly distributed over the genome. Furthermore, possible hot spot integrations were reported as a result of REMI (e.g., Sweigard et al. 1998; Sanchez et al. 1998).

The second effect of REMI on integration events is an increase in single locus integration. Single locus integrations are accompanied by integration of a single vector or by tandem integrations. For example, REMI in *C. graminicola* and *Coniothyrium minitans* resulted in 51 and 8 % single vector integration, respectively (Thon et al. 2000; Rogers et al. 2004). Conversely, the frequency of single locus tandem integration was 20 and 40 %. It is unclear what drives the differences in single vector or tandem vector integrations in REMI. For instance, dephosphorylation of the linearized transforming DNA did not reduce tandem integrations in *Penicillium paxilli* (Itoh and Scott 1997).

26.3 Historical and Modern Usage of REMI

REMI has been used to (1) tag genes by mutational insertion, (2) tag genes by overexpression, (3) tag promoters by green fluorescent protein (GFP), and (4) create stable transformants.

REMI has been mainly used for gene tagging. To this end, a mutant library is created and transformants are screened in a high throughput manner for a particular phenotype. Usually, hundreds to thousands of transformants are screened before a phenotype of interest is found. For this, the increased transformation efficiency caused by REMI is very helpful. The tagging efficiency in REMI can vary between fungi. The percentage of mutants with a tagged gene were 55, 40–67, 50, 30–50, 7–67, and 0 %, respectively in *Fusarium graminearum*, *M. grisea*, *Ustilago maydis*, *Cochliobolus heterostrophus*, *C. cinereus*, and *Hebeloma cylindrosporum* (Seong et al. 2005; Sweigard et al. 1998; Balhadère et al. 1999; Fujimoto et al. 2002; Kahmann and Basse 1999; Lu et al. 1994; Cummings et al. 1999; Makino and Kamada 2004; Combier et al. 2004). Thus, REMI can induce mutations other than insertion of the transforming DNA. The segregation of the selection marker with the phenotype of interest can be assessed when the fungus has a sexual cycle. The phenotype is likely the result of single locus integration when the phenotype and selection marker co-segregate. Sexual crosses can also be used to clear transformants from mutations induced by REMI at other loci.

The insertion site can be identified using plasmid rescue or by PCR based methods like inverse PCR (iPCR) and thermal asymmetric interlaced PCR (TAIL-PCR,[1] Ochman et al. 1988; Liu and Whittier 1995). In the case of plasmid rescue, the genomic DNA is digested with restriction enzymes which do not cut in the vector fragment. The DNA fragments are circularized by self-ligation and transformed to *Escherichia coli* selecting for the bacterial resistance cassette present in the vector that was used for REMI. In the case of tandem integrations, two restriction enzymes can be used of which one cuts in the vector. In this way two plasmids may be rescued each with one of the flanking regions of the genomic insertion. In the case of iPCR, genomic DNA is digested by an enzyme which does not cut the vector. The digested DNA fragments are ligated resulting in circular DNA. Primers binding to the known sequence (e.g., the integrated vector) can be used to amplify the circularized DNA fragment outwards resulting in amplification of the unknown sequence (Ochman et al. 1988). In TAIL-PCR, specific primers that anneal to the integrated vector in the outward direction are combined with degenerate primers in two PCR reactions. High stringency cycles are interlaced with low stringency cycles during the first PCR reaction. In the second PCR the product of the first PCR is diluted and again amplified using high stringency cycles interlaced with low stringency cycles and followed by several normal cycles. This process favors the amplification of a

[1] A detailed protocol on TAIL-PCR can be found in Chap. 46 of this volume

product produced with the specific primer and a degenerate primer (Liu and Whittier 1995). The resulting PCR products are sequenced to identify the location of the vector in the genome. iPCR and TAIL-PCR will be more complex if tandem integrations have occurred. Incidence of sequencing the genomic DNA of tagged mutants will increase, abolishing the need for plasmid rescue or PCR based methods to identify the location of vector integration. To confirm the involvement of the identified integration in the phenotype, the rescued plasmid can be used as a knockout construct for the parental strain. Alternatively, a knockout construct can be created based on the sequenced flanks. If the REMI event is responsible for the selected phenotype, homologous integration of the knockout construct should result in the same phenotype. Complementation of the tagged mutant with an intact copy of the gene is an alternative to confirm that the tagged gene is responsible for the mutant phenotype.

The presence of multiple vector integration sites complicates the identification of the integration event responsible for the phenotype. Southern blot analysis can be used to determine the number of vector integrations if it is not possible to cross mutants with the parental strain to assess the segregation of phenotype and selection marker. In case of multiple vector integration sites the flanking regions of all events should be identified. Each identified gene should be complemented or inactivated in the parental strain to verify the involvement of the candidate gene in the observed phenotype. As mentioned above, a second downside of REMI is the occurrence of mutations unrelated to a vector integration event. For instance, it was shown in *M. grisea* and *F. oxysporum* that tagged genes were not responsible for the observed phenotype (Sweigard et al. 1998; Yoshida et al. 2008). Mutations not related to an integration event might arise if DSB caused by restriction enzymes or other sources are repaired in a processed manner causing small deletions. In such a case, identification of the responsible gene is complicated although the introduction of methods to sequence the genomic DNA or to analysis transcriptomes have made it more simple to identify the gene of interest.

Deletion libraries of *S. cerevisiae* and *Neurospora crassa* are available (Giaever et al. 2002; Dunlap et al. 2007). However, for many fungi such libraries will not be available in the years to come. Here, REMI might be used to create mutant libraries when homologous recombination and RNAi are not efficient or absent in a particular fungal species. If a genome sequence is available, restriction enzymes used for REMI can even be chosen based on restriction sites found in target genes.

Promoter-tagged restriction enzyme-mediated insertion (PT-REMI) is a variant of REMI, which was developed in *Aspergillus niger* (Shuster and Connelley 1999). A vector is used that contains a strong promoter. The vector randomly integrates in the genome and this may result in overexpression or disruption of a gene depending on the integration site. The tagging of genes through overexpression might be useful to activate silent pathways for instance involved in secondary metabolite production. A second variant of gene tagging is the tagging of promoters by GFP. This is achieved by integration of *gfp* lacking a promoter sequence. This approach has been successfully used in *U. maydis* to identify genes upregulated during plant infection (Aichinger et al. 2003). With modern transcriptomics, even going to the single cell level (de Bekker et al. 2011), the use of REMI for this purpose seems obsolete. In specific conditions when it is impossible to isolate RNA, promoter tagging by GFP might still prove useful.

The fourth use of REMI is to create stable transformants. This has been reported in *C. albicans* and *Trichophyton mentagrophytes* (Brown et al. 1996; Kaufman et al. 2004). *C. albicans* transformed by electroporation proved unstable or contained multiple integrations. REMI resulted in stable transformants with mainly single integrations. PEG mediated transformation of *T. mentagrophytes* also proved unstable, while biolistics and AMT did not produce transformants at all. The addition of restriction enzymes during transformation resulted in stable *T. mentagrophytes* transformants, most likely due to the production of DSB and activation of the DNA repair system by the restriction enzyme. REMI is

thus worthwhile to try in fungi recalcitrant to other transformation procedures.

26.4 Alternatives for REMI

Several alternatives for REMI are available: random integration of linear DNA fragments (RALF), transposon tagging, AMT, and phleomycin mediated integration. In RALF, linear DNA fragments containing a selection marker integrate randomly at a single locus without the use of restriction enzymes. This has resulted in thousands of transformants in *H. polymorpha* (Van Dijk et al. 2001) and its use was also described in *C. famata* (Dmytruk et al. 2006). Its efficiency may depend on the dominant DNA repair pathway of the host and the frequency that DSB occurs without addition of restriction enzyme. Transposon tagging has been successfully used in several fungi (Weld et al. 2006; Daboussi and Capy 2003). However, not all fungi have a characterized transposon system available. The development of a heterologous transposon system is not easy. On the other hand, the presence of multiple endogenous transposon elements can complicate the characterization of mutants if an endogenous transposon system is used. Addition of phleomycin during protoplast transformation results in effects similar to REMI in the case of *Schizophyllum commune* (Van Peer et al. 2009). Phleomycin is structurally related to the bleomycin family of antibiotics. It causes double stranded DNA breaks primarily at GC and GT sites (Keith et al. 1987; Hecht 2000). Phleomycin increased transformation efficiency 10-fold when the antibiotic was added during the first 3 h of protoplast regeneration. In addition, single integration events increased from 9 to 55 % (Van Peer et al. 2009). Unlike restriction enzymes the action of phleomycin does not depend on a restriction site. Therefore, it is expected to cleave the genome more random than restriction enzymes. AMT has been developed as a viable alternative to REMI for transformation and gene tagging of fungi (Bundock et al. 1995; de Groot et al. 1998; Weld et al. 2006). A wide range of fungal tissues can be transformed with AMT, ranging from protoplasts, mycelium, conidia to fruiting bodies. AMT can be considered superior to REMI in *C. minitans* since it produces more single-copy integrations randomly distributed over its genome (Rogers et al. 2004). In *Colletotrichum acutatum* REMI did not improve transformation efficiency (Chung et al. 2002). AMT proved superior since a large number of transformants could easily be produced (You et al. 2007; Talhinhas et al. 2008). Furthermore, in *M. grisea* (synonym: *M. oryzae*) mutant phenotypes of REMI transformants were untagged in 33–60 % of the cases (Balhadère et al. 1999; Sweigard et al. 1998). On the other hand, AMT resulted in single integrations in 56–68 % of the cases (Rho et al. 2001). AMT was also used to create a *M. grisea* mutant library based on a high throughput based protocol (Jeon et al. 2007). However, also in AMT the phenotype of mutants can be unrelated to the integration event (Weld et al. 2006; Jeon et al. 2007; Michielse et al. 2009). For example, in *Leptosphaeria maculans* mutants were untagged with a frequency of 50 % (Blaise et al. 2007). This makes complementation of the mutant and the generation of a clean knockout in the parental strain essential, like in REMI. Furthermore, both AMT and REMI result in tandem insertions but the frequency of tandem insertions may be lower in the case of AMT (Rogers et al. 2004).

26.5 Blueprint for REMI

The optimal conditions for REMI can differ substantially depending on the fungal species used. Therefore, a detailed REMI protocol will not be presented. The reader is advised to check Table 26.1 for their fungal species of interest. Table 26.1 provides an indication for the success of REMI of a fungus of interest. The measure of success of REMI is based on the increase in transformation efficiency, frequency of single insertions, successful library formation and mutant identification, its use for goals other than the production of a mutant library and its reported use in literature (++, very successful; +, successful; ±, success of REMI not clear; –, not successful or

an alternative method proved superior). In the case of "++" or "+" it is advised to follow the protocol presented in the publications describing REMI after a critical evaluation for improvements. In the case of "±" and "–" it is advised to critically evaluate the protocol described in the references and possible alternatives to REMI. For example, in the case of *M. grisea*, *C. acutatum*, and *C. minitans* it is clear that AMT is a superior method for the production of mutant libraries. If, however, the reader wishes to pursue REMI it is advised to use the blueprint as a guide for designing and improving the REMI transformation procedure. The blueprint discusses PEG mediated transformation since the majority of REMI transformations reported in the literature are based on this protocol. There are four main variables in REMI that are important for its success. Namely, (1) the form in which the vector is added, (2) the enzyme added during vector linearization and transformation, (3) the enzyme concentration, and (4) the transformation conditions (e.g., temperature and the type of PEG).

26.5.1 REMI Blueprint

1- Prepare protoplasts for PEG mediated transformation.
2- Linearize the vector with the selected enzyme. Make sure this enzyme creates compatible ends with the enzyme added during the transformation procedure.
   a. It is advised to start with *Bam*HI or *Hind*III since these enzymes have been most commonly used during REMI transformations.
   b. A variety of enzymes may be used if a large mutant library is required. In addition, 4-cutters (like *Dpn*II), if generating compatible ends with the enzyme used to linearize the plasmid, might generate a more random integration pattern. To assess if a restriction enzyme is active inside the cell, a reduction in protoplast regeneration should be observed at high enzyme concentrations.
3- Add the restriction mix (containing the linearized vector) to the protoplasts and complement the concentration of the restriction buffer. In the case the concentration of the restriction enzyme in the restriction mix is too high for REMI, inactivate the enzyme or purify the DNA and add restriction enzyme to the protoplasts in the desired concentration.
   a. It is important to try various enzyme concentrations. Start with the addition of 1, 10, 30, and 100 units per reaction. Try additional concentrations of restriction enzyme surrounding the optimum found. If no optimum is found, the extremes (e.g., concentrations lower than 1 U and higher than 100 U) may be assessed.
   b. If *Bam*HI and *Hind*III do not generate an increased transformation efficiency in a variety of concentrations, other enzymes may be tested.
   c. In some cases the addition of restriction buffer in the transformation can result in a reduced transformation efficiency. Therefore, a reaction without buffer may be tried as well. To this end, transforming DNA can be purified prior to addition to the transformation mixture.
   d. Optional: Incubate the protoplasts, DNA, and enzyme for 20 min on ice prior to the addition of PEG solution (see 4.).
4- Add the PEG solution to the protoplasts, DNA, and enzyme. Incubate for 20–30 min on ice.
   a. Initially 2–2.5 % of the total volume of PEG may be added. After 10–15 min incubation on ice the rest of the PEG mixture can be added.
   b. The temperature of incubation may be increased. This should theoretically improve intracellular restriction enzyme activity.
   c. Different types of PEG (e.g., PEG3350-PEG8000) and concentrations (e.g., 40–60 %) may be tested.
5- Add regeneration medium (e.g., 2 % malt extract, 0.6 M sucrose) to the transformation mixture and regenerate the protoplasts. Transfer the regenerated protoplasts to selection medium. Alternatively, transfer the protoplasts to selection medium immediately.
   a. Removal of PEG prior to the addition of regeneration medium may increase transformation efficiency. To this end, 10 volumes

of cold buffer (e.g., STC buffer containing 0.6 M sucrose, 10 mM Tris-HCl pH 7.5, and 10 mM $CaCl_2$) may be added to the transformation mix. Protoplasts can be taken up in regeneration medium after the liquid containing the PEG is removed.

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TALEN-Based Genome Editing in Yeast

27

Ting Li, David A. Wright, Martin H. Spalding, and Bing Yang

27.1 Introduction

Fungi are a diverse group of eukaryotic organisms ranging from unicellular yeasts to filamentous and complex multicellular organisms; some of which can produce fruiting bodies such as the familiar mushroom. They have a world-wide distribution and are found in moderate to harsh environments; both on land and in water. Their life cycle is equally varied, engaging in asexual and sexual reproduction through budding or fission and spore production that results in single or multinucleated haploid and diploid cells. The genome is generally compact. The cell wall contains both glucan and chitin, and these organisms are heterotrophic. In many respects, fungi span the region between plant and animal kingdoms and are equally important in terms of the environment, industry, medicine, agriculture, and science.

Many fungi play a central role in the daily lives of humans. They are used to produce certain chemicals, enzymes, medicines, foods, and their degradative properties are simultaneously useful for bioremediation and are the subject of research aimed at suppression. Certain fungi form symbiotic relationships and others are pathogenic while most are simply prolific recyclers. A few fungi, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Neurospora crassa*, and *Aspergillus nidulans* have become valuable model organisms for scientific study and industrial purposes.

With the advent of high throughput genome sequencing and the many omics programs comes the opportunity for in depth exploration of many other important fungi. Traditionally, this begins with random mutagenesis methods and isolation of mutants, which has yielded significant insights into fungal biology over many decades. Given the enormous potential of the omics era, a more practical approach is to target specific genes using a precise method that allows a choice of deletion, insertion or modification at any given locus with potential as a high throughput method.

We refer to this technology as Genome Editing, which has been in development for about 30 years. A key discovery was that a targeted chromosome double-strand break (DSB) allows a degree of control over the genetic recombination process giving researchers the opportunity to make predetermined genomic modifications. Through its evolution, several different types of nuclease-based technologies have been developed for this method. This chapter will touch on the events leading up to Genome Editing and early technology development, then focus on transcription activator like effector nucleases (TALENs), including their uses, insights gathered from other species, and a TALEN assembly

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protocol and applications in the yeast *S. cerevisiae*. This information is intended to assist with the future development of TALEN technology in a broad range of fungal species.

27.1.1 Overview of Recombination

Cells routinely encounter DSBs as a result of replication processes and external factors. In response, pathways have evolved to efficiently repair DSBs through recombination in order to maintain genome integrity. Recombination-dependent DSB repair can be achieved through either non-homologous end-joining (NHEJ) or homologous recombination (HR). In the NHEJ pathway, repair is dependent upon DNA microhomology of both ends at the DSB point, which is independent of a repair template or donor DNA. If the DSB is simple then repair is made through existing microhomology and is often precise. Alternatively, DNA may be removed and the repair made through new microhomology interactions resulting in small deletions or insertions at the DSB site and culminating in loss, addition, or alteration of genetic material (Fig. 27.1) (Lieber 2010). On the other hand, HR is a repair process that is dependent on a donor DNA template. Once a DSB is detected, the 5′ ends of the break point are resected to expose 3′ single-stranded DNA, which invades a donor DNA through homology. The 3′ end is then used as a primer to copy information from the donor DNA. At some distance, which may be species dependent, extension ends and the newly synthesized DNA strand finds homology with the other free end. Gaps are filled in and the break is sealed through ligation. HR replaces lost information as dictated by the donor DNA, which may result in a gene conversion event if the donor encodes

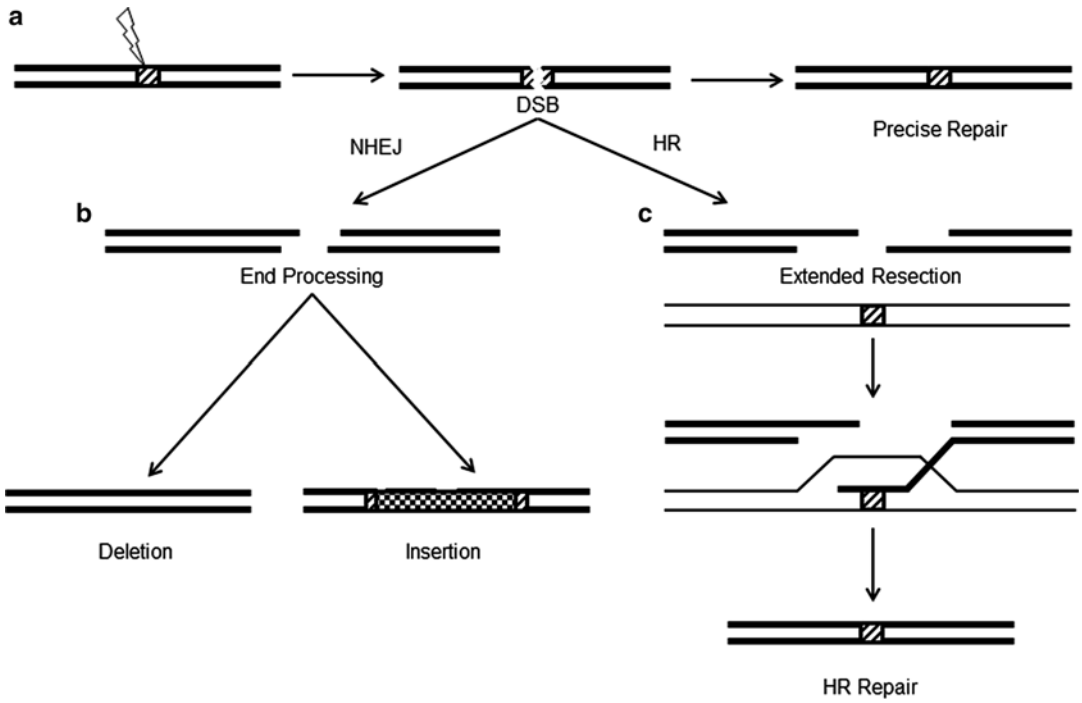

Fig. 27.1 NHEJ and HR repair of a DSB are depicted. The lightening symbol represented a DSB mediator, *double black bars* represent double-strand DNA, and *boxes with stripes* represent the DSB target site while the *checkered box* represents an insertion event. In (**a**) a DSB is created, which generally results in a precise repair through NHEJ. Alternatively, in (**b**) the ends may be resected and repair may result in a deletion or an insertion through NHEJ. In the HR pathway, as shown in (**c**), the resected 5′ ends find homology with a donor DNA that acts as a repair template, lost information is copied, the ends find homology with each other, gaps are filled in, and the break is sealed by ligation

alternative information (Fig. 27.1) (San Filippo et al. 2008). Of the two pathways, HR is the most valuable because it allows directed and specific changes to the genome. This includes large insertions or deletions or single nucleotide insertions, deletions, or modifications, whereas NHEJ is useful for gene knockouts, because it generally leads to a small random insertion or deletion at the DSB site and does not require additional DNA in the form of a donor.

When considering the introduction of exogenous DNA into the genome, either NHEJ or HR is generally dominant in a given species although there may be some variability based on cell cycle or cell type. An example is found in mouse. For most cell types NHEJ is dominant, but embryonic stem cells have an increased HR potential allowing routine production of knockout mice (Porteus 2007). Another example of HR being the preferred pathway can be found in the moss, *Physcomitrella patens*, making it an attractive model organism (Schaefer and Zryd 1997). Nevertheless, the NHEJ pathway is thought to be the most prevalent entryway for exogenous DNA in many species, restricting fine scale modification of the genome, which limits our knowledge base and the commercial potential of most species.

Another exception is the well-studied yeast *S. cerevisiae* in which HR is the dominant pathway for DNA entry into the genome. In fact, early recombination experiments in *S. cerevisiae* account for much of the basic knowledge that current recombination technologies are founded upon. For example, it was found that markers with as little as 20 bp of flanking homologous ends could be directly and precisely incorporated into a gene of interest in this organism with high efficiency, apparently without assistance from a DSB (Hinnen et al. 1978; Orr-Weaver et al. 1981, 1983; Rothstein 1983; Szostak et al. 1983). Work such as this culminated in the various recombination strategies that have been a cornerstone of yeast genetics for decades. Unfortunately, these same techniques do not work well in most other species, and only when mobile introns and other DSB-related cellular processes were studied, meaningful progress was made toward recombination strategies. For example, the *S. cerevisiae* mitochondrial mobile intron replicates through DSB mediated recombination using an encoded meganuclease that generates a single DSB in an intron-less allele of the 21S ribosomal RNA (rRNA) gene. Once the DSB is created, repair is initiated through HR using a mobile intron-containing 21S rRNA gene as a template, resulting in gene conversion from replication of the intron (Dujon 1989). Once this process was understood, it was realized that a targeted DSB may induce recombination in other species, and the encoded I-SceI meganuclease became the inspiration for recombination experiments in mouse, frog, and tobacco cells (Puchta et al. 1993; Rouet et al. 1994; Segal and Carroll 1995). Progress after these initial experiments sparked further interest in studying recombination using nucleases as an initiator of targeted DSBs.

27.2 Genome Editing Technology

Successful use of native meganucleases to mediate targeted NHEJ and HR eventually led to engineered meganuclease and zinc finger nuclease Genome Editing technologies, which have dominated the field for the last decade or so (Klug 2010; Pabo et al. 2001; Stoddard 2011). Unfortunately, implementation of either technology can be cumbersome: nuclease engineering is relatively difficult, many nucleases fail, each technology is limited by the availability of preferred target sites, and both are known to result in much cell death due to off-target DSB induced toxicity issues. However, once a high-quality engineered nuclease pair is obtained, either technology can be very effective. A solution for many of the shortcomings of earlier technologies may be found in the pathogenic bacterial transcription activator like effector (TALE) proteins that are secreted into host cells to support the infection processes (Bogdanove et al. 2010; Scholze and Boch 2011). Decryption of the DNA recognition code for these proteins revealed a simple one-to-one recognition pattern between repeat motifs in the protein and target nucleotides. Since then, TALE proteins have been routinely engineered to make artificial transcription factors and TALE nucleases (TALENs).

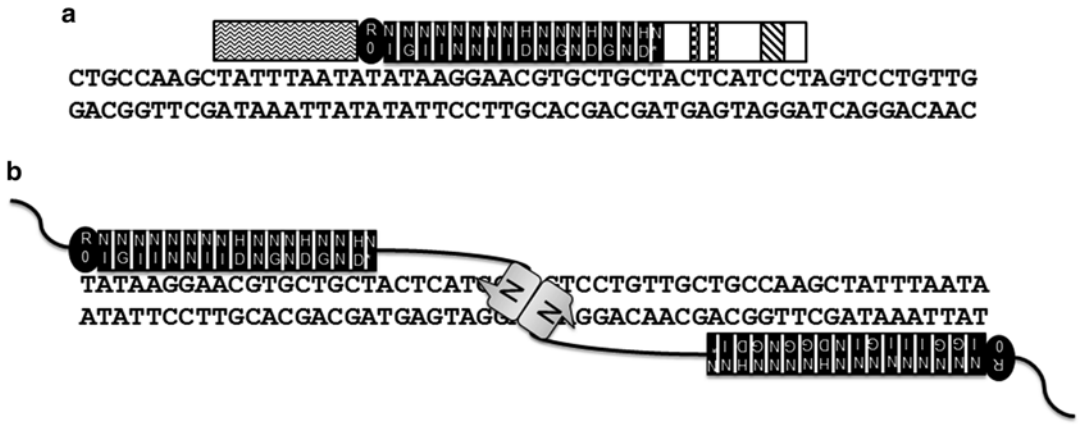

Fig. 27.2 A depiction of a TALE and a TALEN. In (**a**) a TALE is shown bound to its target where R0 specifies a T followed by RVDs for consecutive bases. The box with *wavy lines* represents the N-terminus containing the bacterial secretion and translocation signal and the *white box* encodes the C-terminus nuclear localization signal as suggested by the *small checkered boxes* and the transcription activation domain as shown by the larger box with *diagonal lines*. In (**b**) a TALEN pair is shown bound to their target half sites. The N- and C-termini may be full-length or truncated depending on design. However, each contains a C-terminal FokI fusion as represented by the shaded boxes containing an N

TALE proteins have greatly simplified nuclease engineering due to their truly modular nature and simple DNA recognition code. A TALE is made up of an N-terminal secretion signal, a degenerate helix-turn-helix motif termed repeat 0, a series of repeating units that are up to 33.5 units long, several C-terminal nuclear localization signals, and a transcription activation domain (Fig. 27.2) (Boch and Bonas 2010; Mak et al. 2012). Repeat 0 generally recognizes a T, which is the first base in a target DNA sequence, then each repeat unit, specified by the repeat variable di-residues (RVDs) at the positions 12 and 13, consecutively recognizes successive bases through the end of the target DNA. The code for the most common RVDs is HD, NI, NG, and NN, which predominantly bind to cytosine (C), adenine (A), thiamine (T), and guanine (G) or A, respectively (Boch et al. 2009; Moscou and Bogdanove 2009). To make a TALEN, the repeat 0 and a series of RVDs are assembled guided by the code and the sequence specified by the desired target, then cloned into a scaffold containing the truncated or full-length TALE N and C termini, and a FokI nuclease domain at the C-terminal end. To make a functional nuclease pair, a target is chosen such that two TALENs bind separate targets in opposition to each other and with an appropriate spacer between them. In this orientation, the FokI nucleases can efficiently dimerize and cleave the target DNA (Fig. 27.2). For target site selection, each half site should begin with a 5′ T followed by 12–24 bases reflecting a good mix of nucleotides then a 12–31 bp spacer between half sites. The TALE C-terminal FokI domain fusion point determines the optimal spacer. Compared to the labor intensive methods and high failure rates associated with meganucleases and zinc finger nucleases, TALEN engineering is relatively simple and reliably produces effective nucleases (Briggs et al. 2012; Cermak et al. 2011; Kim et al. 2013; Li et al. 2011; Reyon et al. 2012; Schmid-Burgk et al. 2013). Like zinc figure nucleases, TALEN system requires a pair of nucleases to cause DSB and consequently the genomic editing. Most recently, the CRISPR/Cas-based system has emerged as the new choice of tools for targeted Genome Editing. The simplified derivative of the type II CRISPR/Cas9 systems from *Streptococcus pyogenes* consists of Cas9 nuclease and a single guide RNA (sgRNA), the so called sgRNA/Cas9. The sgRNA/Cas9 has been used for genetic modifications in a variety of organisms including yeast (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013; Sander and Joung 2014; DiCarlo et al. 2013).

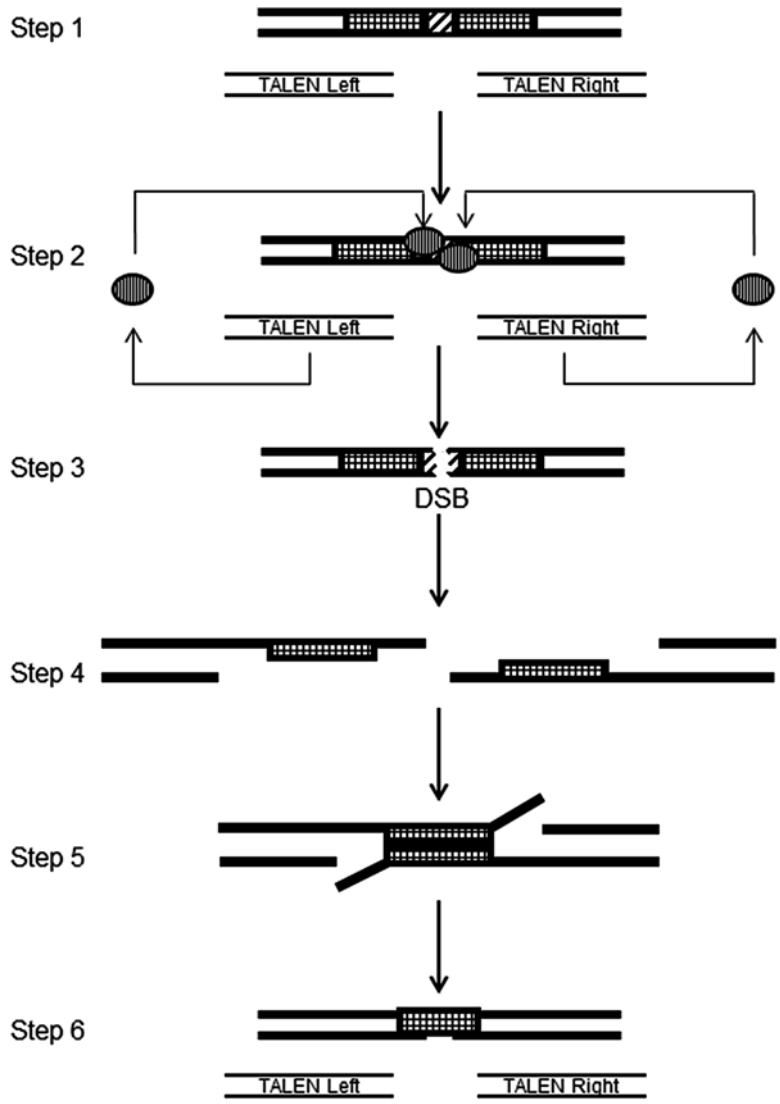

Fig. 27.3 A simplified depiction of the yeast recombination assay. The inactive target gene is shown as heavy *double bars* while the box with *diagonal stripes* and the *rectangles* containing a grid pattern represent the nuclease target sequence and an internal duplication in the inactive marker gene, respectively. The *light double bars* labeled TALEN left and TALEN right depict the TALEN expression plasmids and the oval with vertical stripes is the TALEN protein. In step 1, the inactive marker gene and TALEN plasmids are introduced into yeast cells. In step 2, the left and right TALENs are expressed and the proteins bind the target sequence in the inactive marker gene. In step 3, a DSB is created and in step 4, the 5′ ends of the DSB are resected. In step 5, homology is found between the broken ends at the internal duplication sites of the inactive marker. In step 6, unmatched DNA is removed, gaps are filed in, and the break is sealed to restore function to the marker gene

Regardless of which engineered nuclease technology is pursued, all three work in a similar manner. A genomic target site is chosen based on known parameters for each system, then a nuclease pair is engineered and tested in a cell-based assay such as the yeast recombination assay (Fig. 27.3). Once a suitable nuclease pair is obtained, the pair is cloned into an expression vector for delivery to the cells of the intended species. At this point the process diverges

depending on weather NHEJ or HR mediated Genome Editing is the objective. For NHEJ, the engineered nuclease pair is transformed into the species of interest. The pair is expressed in the cytoplasm and then moves to the nucleus where it binds the target and generates a DSB. The cellular repair process senses the DSB and repairs are made, resulting in a fraction of cells with site-specific mutations. If the expected phenotype is selectable or screenable then putative knockouts are examined for the expected mutation. If the phenotype is not amenable to selection or screening, then a selectable marker gene can be included with the nuclease pair. The marker allows selection of transformed cells, which can then be effectively screened for the desired mutation. For HR, the engineered nuclease pair is transformed into cells along with a donor DNA that has homology to the target and carries desired changes to be incorporated into the locus. The nuclease pair binds to the target, generates a DSB, followed by resection, leaving free 3′ DNA that invades the donor DNA, wherein genetic information is copied. Once the extension process ceases, the 3′ ends find homology with each other, gaps are filled and the break is sealed. If the mutation is selectable or screenable, putative mutant cells are assayed for the desired change. If the phenotype is not screenable or selectable, a marker can be added, separate from or included in the donor DNA, to select transformed cells, which are then screened for the desired mutation.

Screening is generally performed using either the T7 endonuclease or Cel1 (Surveyor) assay, although other methods, such as PAGE, RFLP analysis, high resolution melt analysis (HRMA), and the heteroduplex mobility assay (HMA), have been suggested in the literature (Ansai et al. 2014; Dahlem et al. 2012; Hu et al. 2013; Kim et al. 2009; Miller et al. 2007; Oleykowski et al. 1998; Wei et al. 2013). As an example, the T7 endonuclease assay depends on the generation of a DSB at the point of nucleotide mismatch in a double stranded DNA sample. PCR primers are designed so the expected mutation site is centered in a 200–1,000 bp product. The target site from a putative mutant and a known wild-type sample are PCR-amplified independently. Approximately, 200 ng of each PCR product are mixed, melted, and annealed to each other, followed by addition of T7 endonuclease I. The sample is then separated on an agarose gel. If the putative mutant has a base change, the sample will have a prominent uncut PCR product with two lighter bands at the expected size for cleaved products. A PCR sample from the putative mutant is then sequenced to verify the change, followed by additional assays, such as Southern blot analysis, to confirm the expected mutation and the lack of localized genome rearrangements.

27.3 TALEN Uses and Considerations

As indicated above, nuclease-mediated Genome Editing technology is generally used for the production of small targeted insertions, deletions, or base modifications at or near the DSB site. These fine scale alterations are relatively common; however, larger scale mutations have been reported in the literature when two nuclease pairs are used simultaneously. These include large deletions, inversions, and translocations. Examples include a 5.5 megabase deletion in zebrafish, an inversion in pig fibroblast cells and translocations generated in human cells (Carlson et al. 2012; Gupta et al. 2013; Piganeau et al. 2013). Whether fine or large scale, the technology can be used to modify genes of known function, identify the function of unknown genes, knockout large and small RNAs, disrupt gene clusters, and study genome rearrangements that lead to conditions such as cancer.

Another use of the technology is gene activation or repression through the production of artificial transcription factors. Here, the FokI nuclease portion of a TALEN is replaced by an activator or repressor domain, which may simply mean retention of the endogenous TALE activation domain as an activator. TALE transcription factor binding sites are generally chosen in proximity to the TATA box in the promoter of the gene of interest, but have been reported to function as far as 500 bp upstream (Maeder et al. 2013; Miller et al. 2011). It is thought that a TALE activator binds to its target and effectively changes the site of transcription

initiation while up-regulating mRNA production (Boch and Bonas 2010; Hummel et al. 2012). In the case of the TALE repressor, transcription can be suppressed either by simply binding the target and interfering with RNA polymerase initiation or by addition of a repressor domain (Cong et al. 2012; Crocker and Stern 2013; Mahfouz et al. 2012; Politz et al. 2013). It should be also noted that multiple TALE transcription factors targeted to different locations in a single promoter can act synergistically (Maeder et al. 2013; Perez-Pinera et al. 2013).

There are few citations for application of TALEN or other nuclease-based technologies to fungi other than *S. cerevisiae*. As such, there are many unknowns relating to Genome Editing in fungal species. However, much insight can be drawn from the literature concerning applications in other kingdoms. TALENs have been delivered as DNA, RNA, and protein using a variety of methods such as electroporation, *Agrobacterium* infection, lipofection, micro injection, and biolistic, PEG/calcium chloride or lithium acetate-based methods. Delivery to fungal species may not be an issue since all of these methods have been applied to various fungi (Hinnen et al. 1978; Arnau et al. 1988; Bailey et al. 1993; Chen et al. 2011; Djulic et al. 2011; Herzog et al. 1996; Judelson et al. 1991; Kamoun 2003; Malardier et al. 1989; Mort-Bontemps and Fevre 1997; Olmedo-Monfil et al. 2004; Partida-Martinez et al. 2007; Robinson and Sharon 1999; Ruiz-Diez 2002; Utermark and Karlovsky 2008; Vieira and Camilo 2011). However, the type of molecule to use should be carefully considered along with the intended outcome through either NHEJ or HR.

If using DNA, parameters such as promoter strength, individual promoters versus a single promoter strategy, codon optimization, FokI nuclease domain type, and inclusion of all elements in a single plasmid or use of multiple plasmids should be considered. There is evidence to support the strategy of expressing TALEN pairs for only a relatively short period of time; however, inducible promoters or small molecule control systems are not always available (Porteus and Baltimore 2003; Pruett-Miller et al. 2009). It should also be noted that under-expression may lead to limited activity, while overexpression may be a source of toxicity. Additionally, it is possible to use a single promoter with both TALENs expressed in a single ORF separated by a T2A signal that functions to physically split the two TALEN proteins during translation (Zhang et al. 2013). For some species, codon optimization may be necessary, depending on the codon bias of the TALEN source material, yet TALENs have been used successful in many species without this consideration.

The type of FokI nuclease domain used can have a dramatic effect on the experimental outcome. The native FokI nuclease domain functions as a homodimer, allowing three different types of TALEN interactions. Two left TALENs or two right TALENs can interact to form a functional nuclease, just as a left and a right TALEN can, which can result in greater toxicity. To mitigate against this effect, various FokI heterodimers have been constructed that limit interactions to just a left and a right TALEN. However, many heterodimers also reduce the effectiveness of the nuclease, so there is a tradeoff between activity and toxicity (Miller et al. 2007; Doyon et al. 2011; Guo et al. 2010; Sizova et al. 2013; Szczepek et al. 2007). A TALEN pair that is not particularly toxic to a specific organism may benefit from the FokI homodimer form, but, if one or both TALENs prove toxic, a heterodimer FokI may be necessary to help ensure success of the experiment.

When planning an HR experiment, the donor DNA design may be crucial. The chance of incorporating changes dictated by a donor DNA may decrease with relative distance from a DSB, and this may vary from species to species. For instance, human and mouse cells may have an effectively narrow window of less than 100 bp, while tobacco may have a relatively broad window of around 1.5 kb (Fig. 27.4) (Elliott et al. 1998; Lee et al. 2012; Porteus 2006; Townsend et al. 2009). There are reports describing the distance variable, but a general reduction in incorporation of a donor DNA guided change should be expected with increasing relative distance from the DSB. The optimal length of homology between the target gene and donor DNA is also

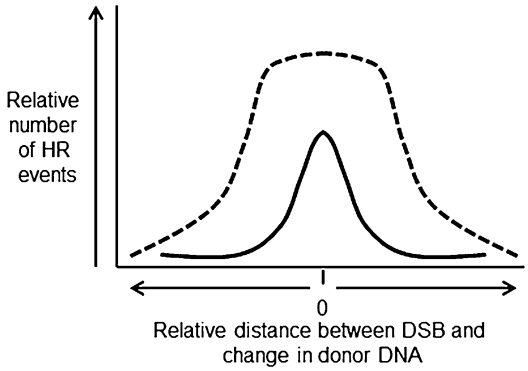

Fig. 27.4 An idealized window of opportunity is depicted. In general the greatest opportunity for HR may exist when the DSB and the desired change encoded by the donor DNA are relatively close to each other. Additionally, in some species, the HR may fall off sharply as relative distance increases as suggested by the *solid line* (mouse) or may extend to greater a distance as suggested by the *dashed line* (tobacco). More data is required before any real conclusion can be drawn

largely unknown. Reported donor DNAs have been larger than 4 kb or as small as 800 bp, or even down to oligo lengths. Unless a size range is established for a species of interest, longer may be a better choice than shorter. One additional point, that may be counterintuitive, is that some evidence suggests that modification of the engineered nuclease target site, through conservative base changes, in the donor DNA may not be necessary. Some reports show only a slight reduction in HR events between donors with and without the target site (Porteus 2006; Townsend et al. 2009; Urnov et al. 2005). This observation may be important when a limited number of changes are desired in a gene of interest, since altering a TALEN target site could involve many base pair modifications, which may adversely affect gene function or otherwise add uncertainty to an experiment.

The use of RNA has several distinct advantages over DNA. For example, (1) RNA does not persist in the cell, which may be important if a non-transgenic strain is desired, (2) Unlike DNA, RNA itself is not known to be mutagenic when introduced into cells, and (3) RNA provides a short burst of nuclease activity, which may be less toxic and lessen the chance of unintended genomic mutations in survivors. Many of the considerations for DNA strategies are relevant to RNA such as codon optimization, use of a T2A element and choice of the FokI domain. Additional considerations include the greater susceptibility of RNA to degradation and the need to generate RNA with a 5′ cap and a 3′ poly A tail and in sufficient quantities for a planned experiment, all of which may add a level of difficulty.

Finally the use of engineered nuclease protein has been reported with or without a cell membrane penetrating peptide. For example, TALEN proteins bearing R9 (poly arginine) or TAT (from HIV) motifs are taken up by human cells to efficiently mediate NHEJ (Liu et al. 2014; Ru et al. 2013). Additionally, protein can be microinjected into some cell types. If protein delivery is considered as an option, it should be first determined whether membrane penetrating peptides or micro injection will work in the species of interest before proceeding. Also, the choice of which FokI domain to use is still an important issue, along with a consideration of the skills necessary to generate active TALEN proteins for any given experiment.

27.4 TALEN-Mediated Yeast Transformation and Genetic Modification Protocol

27.4.1 Materials

27.4.1.1 Yeast Strains

(a) YPH499 (MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1)-haploid
(b) YPH500, isogenic strain to YPH499 but different mating type (MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1)-haploid
(c) YPH500a, isogenic strain to YPH500 but containing a functional *URA3* gene (MATα lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1)-haploid

27.4.1.2 Yeast Growth Medium

(a) YPAD medium: 6.0 g yeast extract (Difco), 12.0 g peptone (Difco), 12.0 g glucose, 60 mg adenine hemisulfate, 10.0 g Bacto-agar

(Difco), and 600 mL distilled water. Autoclave for 15 min in a liquid cycle.
(b) Synthetic complete drop-out (SC drop-out) medium: 4.0 g Difco yeast nitrogen base (without amino acids), 12.0 g glucose, 0.5 g SC drop-out mix, 10.0 g Bacto-agar (Difco) and 600 mL distilled water, pH 5.6. Autoclave for 15 min in a liquid cycle.
(c) 100×5-Fluoroorotic Acid (5-FOA) solution stock: 1 g of 5-FOA in 10 mL of DMSO for a final concentration of 100 mg/mL.
(d) 5-FOA medium: 4.0 g yeast nitrogen base (without amino acids) (Difco), 12.0 g glucose, 0.5 g SC drop-out mix, 10.0 g Bacto-agar (Difco), and 600 mL distilled water, do not adjust pH. Add 6 mL of 100×5-FOA stock after autoclaving 15 min and cooling down to ~65 °C, mix and pour plates.
(e) YPAD-G418 medium: 6.0 g yeast extract (Difco), 12.0 g peptone (Difco), 12.0 g glucose, 60 mg adenine hemisulfate, 10.0 g Bacto-agar (Difco), and 600 mL distilled water. Autoclave for 15 min in a liquid cycle. Cool down to ~65 °C, add 600 μL of 1000× G418 (200 mg/mL) stock, mix and pour plates.

27.4.1.3 Yeast Transformation Reagents

(a) Single-stranded carrier DNA (2 mg/mL)
(b) Lithium acetate stock solution (1.0 M)
(c) Polyethylene glycol 3,350 (PEG 50 % w/v)
(d) Plasmid DNA

27.4.1.4 Yeast Protein Extraction

(a) Cracking buffer stock solution: 48 g Urea, 5 g SDS, 4 mL of 1 M Tris–HCl (pH6.8), 20 μL of 0.5 M EDTA, and 40 mg Bromophenol blue in a volume of 100 mL.
(b) 100x PMSF: 0.1742 g PMSF in 10 mL of isopropanol, stored at −20 °C.
(c) Cracking buffer working solution: A mixture of 10 μL β-mercaptoethanol, 50 μL of 100x PMSF, and 1 mL of cracking buffer stock solution.

Note 1: prepare the working solution just before use.

Note 2: Add additional 1 μL of 100x PMSF stock per 100 μL of cracking buffer every 15 min. The half-life of PMSF in solution is short (about 7 min).

(d) Glass beads, acid-washed 425–600 μm (Sigma-Aldrich).

27.4.1.5 Yeast Genomic DNA Extraction Reagents

(a) Glass beads, acid-washed 425–600 μm (Sigma-Aldrich)
(b) TE buffer (10 mM Tris, 1 mM EDTA, adjust pH to 7.0 with HCl)
(c) Phenol: chloroform: isoamyl alcohol (25:24:1) (Fisher Scientific)
(d) Chloroform (Fisher Scientific)
(e) Isopropanol (Fisher Scientific)

27.4.1.6 Yeast Single Strand Annealing (SSA) Assay

(a) Yeast β-galactosidase assay kit (Thermo Fisher Scientific, Rockford, IL, USA).
(b) Whatman No. 5 filter paper (GE Healthcare, PA, USA).
(c) Z buffer: $Na_2HPO_4{\cdot}7H_2O$ 16.1 g/L, $NaH_2PO_4{\cdot}7H_2O$ 5.50 g/L, KCl 0.75 g/L, $MgSO_4{\cdot}7H_2O$ 0.246 g/L. Adjust pH to 7.0 and autoclave.
(d) X-Gal stock solution: Dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL, GOLD BIOTECHNOLOGY, MO, USA) in *N*,*N*-dimethylformamide (DMF) at 20 mg/mL. Stored in dark at −20 °C.
(e) Z buffer/X-Gal solution: 100 mL Z buffer, 0.27 mL β-mercaptoethanol (Sigma), 1.67 mL X-Gal stock solution.
(f) Liquid nitrogen.
(g) Synergy HT multi-mode microplate reader (Bio-Tek).
(h) BioPhotometer (Eppendorf).

27.4.1.7 Molecular Cloning

(a) TALE assembly plasmid kit [as described (Li and Yang 2013)]
(b) Restriction enzymes—*BsmBI*, *BglII*, *SpeI*, *ScaI*, *BamHI*, *SphI*, *PstI*, *BsrGI*, and *AatII* (e.g., Fermentas)
(c) RNase A (e.g., Fermentas)

(d) 1 kb DNA Ladder (Invitrogen)
(e) Bacterial competent cells
(f) Luria-Bertani (LB) broth medium with appropriate antibiotics, ampicillin (100 mg/L), kanamycin (50 mg/L), tetracycline (20 mg/L)
(g) T4 DNA ligase (e.g., NEB)
(h) GENECLEAN III Kit for DNA purification (MP Biomedicals)
(i) ExoSAP-IT for PCR product cleanup (Affymetrix)
(j) Apparatus for DNA electrophoresis
(k) 30 and 37 °C incubators
(l) 30 and 37 °C shakers

27.4.2 Methods

27.4.2.1 TALEN Constructions

The TALEN central DNA binding domain is a highly repetitive region that is difficult to assemble using PCR-based "stitching" methods. A convenient and rapid Golden-Gate-based cloning strategy was adopted to assemble intermediate repeat arrays followed by final construction in a TALEN scaffold vector. The TALEN version used in this protocol is the full-length TAL effector fused to a homodimeric FokI cleavage domain at the C-terminus. A detailed TALEN construction method was described in our book chapter and the Methods paper (Li and Yang 2013; Umezu et al. 1971). Briefly, the first step is to select the target site ranging from 16 to 23 bp for each of two sub-sites that are separated by an 18–20 bp optimal spacer region. Each sub-site is preceded by thymine at position 0, followed by a good mix of bases, and there is no DNA composition requirement for the spacer region. The second step is to utilize our Golden-Gate cloning library to construct intermediate repeat arrays that each contains eight or fewer repeats. The library is composed of 52 plasmids, including one destination plasmid and 44 pGEM-T-based plasmids each containing a single repeat unit. The repeats were made from four core repeats with RVDs NI, NG, HD, and NN recognizing nucleotides A, T, C, and G, respectively, and with different cohesive ends flanked by the type IIs restriction enzyme *BsmBI* recognition sites. The full TALE repeat region is made from 2 to 3 intermediate repeat constructs, with individual repeats designed to occupy a particular position in each intermediate repeat construct as dictated by the target sequence (Fig. 27.5). To assemble an array of intermediate repeats, eight or fewer of the core repeat plasmids and the destination plasmid are mixed together into one reaction and treated with *BsmBI* for 1 h at 55 °C, then the reaction is cooled down to 37 °C and 1 μL of T4 DNA ligase and 1 mM ATP are added. The reactions are cycled between 37 and 16 °C for 50 cycles. Once the intermediate repeat constructs (first 8mer, second 8mer, plus third 2mer to 8mer if necessary) are assembled, they are transformed into *E. coli* and candidate clones are sequenced. Restriction enzyme sites located at the ends of each repeat array are used to release the arrays, which are further assembled into the TALEN backbone or scaffold vector.

27.4.2.2 Yeast TALEN Expression Vector Construction

TALENs for a pair are cloned into two separate yeast expression vectors: pCP3M and pCP4M, which are modified from the plasmids pCP3 and pCP4. These shuttle vectors both replicate in *E. coli* and in *S. cerevisiae*. pCP3M is about 5.5 kb, consisting of the following major components: yeast TEF promoter, a multiple cloning site (MCS), NOS terminator, a modified yeast *His3* gene (selectable marker), the yeast CEN6/ARS (yeast centromere and autonomously replicating sequence), a modified beta lactamase gene (ampicillin resistance) and the ColE1 replicon. pCP4M contains components similar to pCP3M, except that the yeast *His3* gene is replaced by the *LEU2* selectable marker gene. Complete TALEN coding regions are moved into yeast expression vectors as described (Umezu et al. 1971). Briefly, pCP3M or pCP4M is linearized with *BamHI* and *SpeI*, which are located in the MCS, then constructs encoding full TALEN RVD arrays are digested with *BglII*, *SpeI*, *and ScaI* and the TALEN repeats are moved into pCP3M or pCP4M.

Note 3: *BglII* and *BamHI* generate compatible cohesive ends. And *ScaI* can be used to cut the

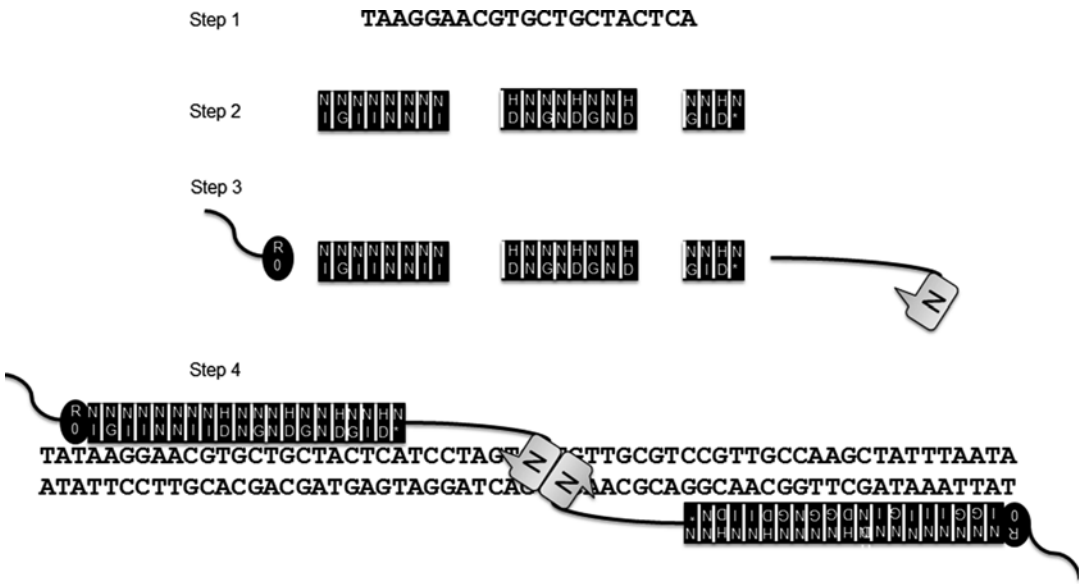

Fig. 27.5 TALEN assembly is depicted. Step 1, choose a target. Target half sites should begin with a T and contain a good mix of bases avoiding stretches of a single base or overly GC or AT rich sequence. The spacer between target half sites should be 18–20 bp. Step 2, using the Golden-Gate cloning method combine plasmids encoding RVDs for the target in sets of eight or less using the target sequence as a guide moving 5′–3′. Remember that R0 recognizes the first T and that R0 is encoded by the TALEN back bone vector so begin assembly starting with the second base of the target half site. Once RVD arrays are assembled, they should be sequenced to ensure proper assembly. In step 3, sequence verified RVD arrays are cut from their assembly vector and a full TALEN is constructed by ligating the arrays into the TALEN back bone vector. Step 4 depicts an assembled TALEN pair bound to their target half sites and the FokI nucleases forming a dimer in the spacer region

backbone of the TALEN plasmid into two smaller pieces for easy separation from the repeat-containing fragment in an agarose gel.

27.4.2.3 TALEN-Mediated Gene Knockout Through NHEJ

Site-specific nucleases are widely adopted tools to induce targeted DSBs for gene modification and frame-shift mutations through NHEJ at predetermined loci. As a proof-of-concept, TALEN technology has also been successfully applied in *S. cerevisiae* to efficiently knockout several marker genes that can undergo either positive or negative selection, or that generate easily screened mutations through visual inspection (Li et al. 2011). Here we describe the protocol for performing *S. cerevisiae* gene knockout using the *URA3* gene as one example. 5-FOA, a specific inhibitor for functional *URA3*-containing strains, is used for negative selection. *URA3* encodes orotidine 5-phosphate decarboxylase (ODCase), which is an enzyme involved in pyrimidine ribonucleotide synthesis (Boeke et al. 1984). ODCase converts 5-FOA into 5-fluorouracil, a toxic compound that kills cells containing a functional *URA3*, only allowing *ura3* deficient cells to grow (Rose and Winston 1984).

Detection of TALEN Expression in Yeast

Transform individual engineered TALEN genes into yeast YPH500a, a strain with a functional *URA3* gene restored from the Ty transposon-disrupted *ura3-52* mutation (Li et al. 2011; Gietz and Woods 2002), using the yeast high efficiency transformation protocol with LiAc/SS-carrier-DNA/PEG (Li and Yang 2013). Also transform empty yeast expression vectors (either pCP3M or pCP4M) as the respective negative controls. Spread yeast cells on SC-His or SC-Leu plates. Extract yeast protein by the Urea/SDS method modified from the Yeast Protocols Handbook (Clontech). Briefly, the modified protocol includes the following steps:

1. Pick three individual colonies to initiate 5 mL overnight cultures in appropriate SD selection medium. Then add these cultures to 50 mL medium and continue to grow until an OD600 of around 0.6 is achieved. Calculate the total number of units by multiplying the OD600 by the culture volume.

Note 4: handle the samples on ice for all steps of the following extraction procedure.

2. Collect the cell pellet by centrifugation at 1,000x g for 5 min at 4 °C. Pour off the supernatant, and wash the cells once with ice-cold water, centrifuge again and freeze the cell pellet at −70 °C until use.
3. Prewarm the cracking buffer to 60 °C. Add 100 μL buffer per 7.5 units of cells (as calculated in step i). Resuspend the cells quickly; incubate the suspension at 60 °C briefly in a water bath to accelerate the suspension if necessary.
4. Transfer the cell suspension to a 1.5 mL microcentrifuge tube that contains 80 μL of glass beads per 7.5 units of cells (as calculated in step i).
5. Vortex the samples vigorously for 1 min.
6. Centrifuge at top speed for 5 min at 4 °C.
7. Transfer the supernatant to a new tube, and heat the sample for 3–5 min. at 100 °C.
8. Perform a Western Blot to measure the TALEN protein expression level by probing with antibody against the FLAG epitope tag that was constructed at the upstream of transcription activation domain.

Screening for phenotypic mutants

1. Transform the paired TALENs into YPH500a.
2. After about 3 days of incubation, resuspend yeast colonies containing the TALEN constructs in sterile water, and measure the cell concentration.
3. Spread about 1×10^5 yeast cells on 5-FOA plates (selection for *ura3* mutant cell) and about 1×10^3 yeast cells on YPAD plates (for estimating the survival ratio). Setup a negative control with constructs lacking the TALEN genes, and also spread 1×10^6 and 1×10^3 of these negative control yeast cells on 5-FOA and YPAD plates, respectively.

Note 5: Usually 1 mL of OD600 0.1 yeast culture contains approximately 1×10^6 cells.

4. After 3 days, count the colony numbers on the 5-FOA and YPAD plates, separately. Calculate the mutagenesis efficiency using the following equation: Total number of 5-FOA resistant colonies/total number of YPAD colonies = % mutation efficiency. Divide the negative control efficiency by ten. Replicate this experiment twice, with measurements performed in triplicate.

Note 6: In this experiment, the actual mutation rate should be higher than the observed ratio. This may be due in part to the screening method, which selects for an auxotrophic phenotype with a knockout genotype. The *URA3* gene containing in frame insertions/deletions might still be functional and confer lethality to the yeast cells in the 5-FOA medium.

Confirming the Mutant Genotypes

Whether 5-FOA resistant colonies contain the desired mutations generated by correspondent TALENs needs to be further validated.

1. Pick several 5-FOA tolerant colonies and re-streak them on 5-FOA plates and grow for 3 days.
2. Extract genomic DNA from individual yeast clones. Briefly, scrape off the cells from plates and wash them once in distilled water, centrifuge and remove the wash water.
3. Add 200 μL TE buffer to resuspend the cells, add 1/3 volume of acid-washed glass beads and 200 μL phenol: chloroform: isoamy alcohol (25:24:1).
4. Vortex the mixture at the highest speed for 5 min, and centrifuge at 14,000 rpm for 10 min.
5. Transfer the aqueous (upper) phase to a new tube, add 200 μL of chloroform, shake the tube vigorously for 30 s, and centrifuge for 10 min at 14,000 rpm.
6. Transfer the aqueous (upper) phase again to a new tube and add 4/5 volume of isopropanol to precipitate the DNA by centrifuging at 14,000 rpm for 10 min.
7. Wash the DNA pellet with 500 μL of 70 % ethanol.

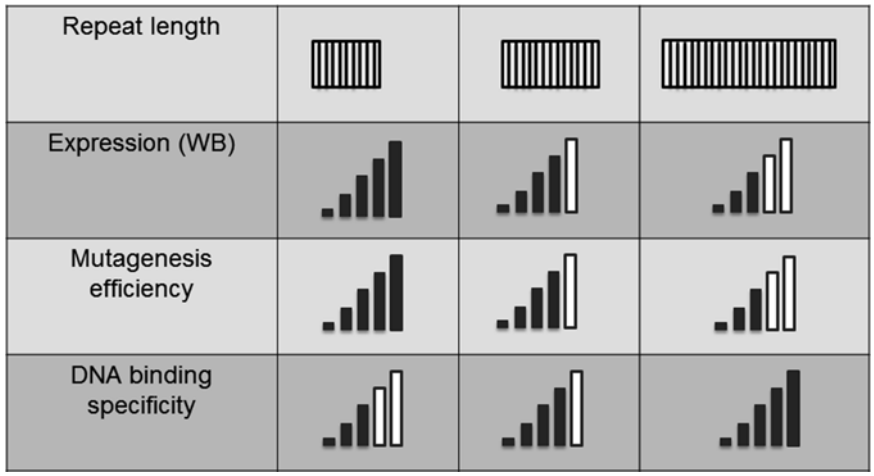

Fig. 27.6 The correlation among repeat length with expression level, mutagenesis efficiency, and DNA binding specificity of designer TALENs. In the repeat length row, the rectangle represents the TALE central repeat part. Different rectangle number means different length of repeat region. The left graph with fewest rectangles means the shortest repeat region, as such, the middle one is the medium length, the right graph with most rectangles is the longest repeat length. For the expression level, the full signal strength represents highly expressed proteins when detected by Western Blot. Signal strength with one *white color* filled bar means expressed at comparatively lower level. And the one with two *white color* filled bars is the relatively lowest expression. For the mutagenesis efficiency and DNA binding specificity, in the similar way, higher signal strength means higher mutagenesis efficiency or stronger DNA binding specificity, and less signal strength shows lower mutagenesis efficiency or weaker DNA binding specificity. So the repeat length determination is further dependent on the specific application requirements and main purpose

8. Briefly dry the pellet and add 30 μL of TE buffer to dissolve the DNA.
9. Use 1 μL DNA as template and gene-specific primers to PCR-amplify a ~500 bp DNA region centered at the targeted site. Treat the PCR product with ExoSAP-IT for 15 min at 37 °C to remove unconsumed primers and dNTPs, followed by heating the reaction at 80 °C for 15 min to inactivate the ExoSAP-IT enzyme. Sequence the samples to confirm the mutant genotypes.

Note 7: The gene disruption frequency generated by TALENs can reach up to 10^{-2} (1 in 100). Our sequencing data revealed that the most frequent mutations are small deletions, but with one mutant containing a deletion of 168 bp. Among the deletion genotypes many contained several bp of microhomology at the junction. Some mutations did result from a one or two bp insertion, but base substitutions were rare.

Note 8: Based on the observation of TALEN performance in yeast, it appears that expression and mutagenesis efficiencies are inversely proportional to the repeat number in engineered TALENs. Western blot results indicate that TALENs with more repeats have a lower expression level relative to TALENs with fewer repeats (data not shown). TALEN-mediated mutagenesis efficiency was notably higher for the shorter TALEN pairs. Theoretically TALENs with longer repeats may increase DNA recognition specificity, but at the same time their expression may be compromised (Fig. 27.6). It should also be noted that TALENs with shorter repeats did not show an increase in toxicity compared to longer TALENs, as might be expected. However, the significance of these observations is unclear, and a thorough examination of this correlation needs to be completed before any concrete conclusions can be drawn.

27.4.2.4 Gene Knock-In Through HR

In yeast, the error-prone NHEJ efficiency is much lower than homologous recombination. When the mutation of a gene of interest is not phenotypically selectable, it is difficult to detect gene knockout mutations generated through the NHEJ repair pathway. On the other hand, gene knock-in through HR may make DNA mutagenesis screening easier and less laborious by using a donor DNA containing a selection marker, such as a neomycin phosphotransferase II (*NPTII*) expression cassette located between the homologous arms. The frequency of HR-based mutagenesis or gene knock-in is enhanced dramatically by the TALEN-induced DSBs. For example, the rate of HR-based restoration of *URA3* function is about 4.5–34 % vs. 0.001–0.1 % of control dependent on different TALENs used (Li et al. 2011). The homologous arm length can range widely, from dozens to hundreds of bp, even to several kb on each side.

1. Donor DNA preparation. PCR-amplified and purified donor DNA can be directly transformed into yeast, or the PCR product can also be cloned into a subcloning vector such as pGEM-T that does not replicate in yeast. Ideally, linearize and purify the plasmid DNA before use.
2. Transform the donor DNA plus the target gene TALEN pair or the donor DNA plus the empty vectors (negative control) into YPH500a and select on SC-His-Leu medium.
3. After 3 days, pick dozens of colonies and spread 1×10^4 cells of each on appropriate selection medium, such as YPAD-G418 medium (for *NPTII* selection). At the same time, plate 1×10^3 cells on YPAD medium for survival rate calculation. Additionally, screen 1×10^6 negative control cells containing the empty yeast expression vectors and donor DNA also on YPAD-G418 medium.

Note 9: Usually the HR efficiencies can reach up to 10^{-1} (1 in 10) when using the autonomous donor cassette as a selection marker, and the negative control frequency is about 10^{-3} (1 in 1,000), which is probably caused by basic HR events in yeast.

27.4.2.5 Yeast Single Strand Annealing (SSA) Assay for Transient and Rapid Evaluation of Nuclease Activity

TALEN-mediated yeast genome modification has great potential for exploration of basic genetic mechanisms. Additionally, yeast is an ideal model for TALEN activity estimations using a SSA assay. The reporter construct, pCP5, is made using a nonfunctional *LacZ* gene containing a nuclease target cloning site located between a 125 bp internal duplication of the *LacZ* gene. In detail, pCP5 contains the following main elements that are essential for the SSA assay and for both *E. coli* and yeast replication and selection: yeast GPD promoter, *E. coli LacZ* 5′ half, 125 bp internal duplication sequence, modified yeast *URA3* gene, *ccdB* gene and chloramphenicol acetyltransferase (*CAT*) gene, 125 bp internal duplication sequence, *E. coli LacZ* 3′ half, modified *nptIII* gene (for *E. coli* antibiotic selection), modified pBBR replicon (medium copy number), yeast 2 μm replicon, and yeast *Trp1* gene (for yeast selection marker).

Once transformed into yeast, functional TALENs generate a DSB at the target site in the *LacZ* gene, and then the repair enzymes generate free 3′ ends that find homology with each other in the duplicated region. Unpaired ends are removed, gaps are filled in, and the break is sealed, which generates a functional *LacZ* gene that can be assayed, and the results are used as a way to estimate TALEN activity (Fig. 27.7).

Note 10: pCP5 is a high copy plasmid in yeast and a medium copy plasmid in *E. coli*, which should be grown in LB with 15 mg/mL of kanamycin.

Construct SSA Assay Plasmids

1. For this assay, the yeast expression vectors, pCP3M, pCP4M, are the same as the vectors used for the genome modification experiments, TALEN genes can be moved from intermediate vectors to yeast expression vectors.
2. Digest the reporter plasmid, pCP5 as described above, with *BglII* and *SpeI* to release 2.5 kb of the *ccdB* gene and the *CAT* gene.

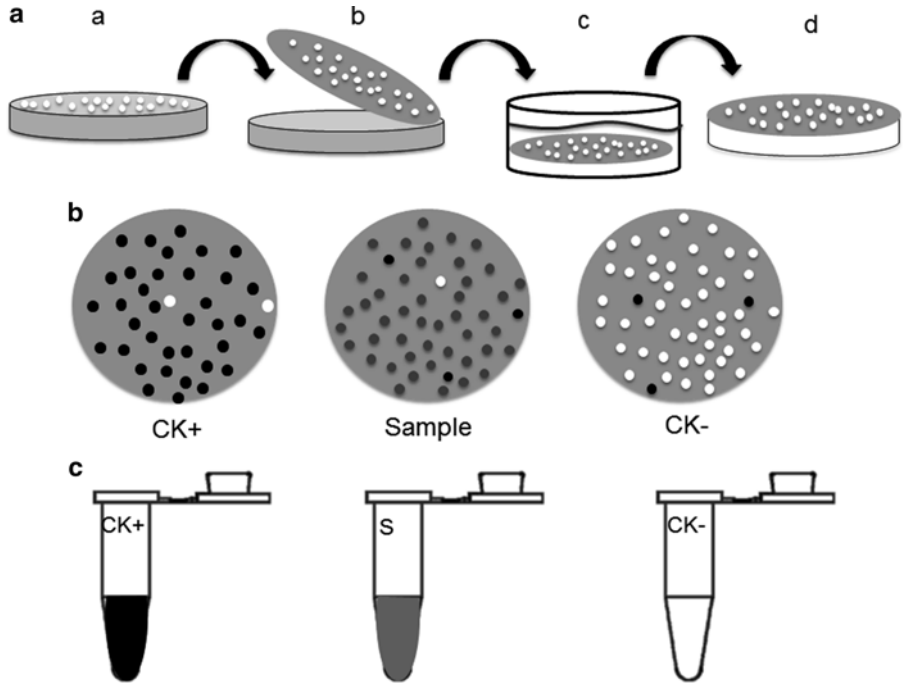

Fig. 27.7 Depiction of yeast SSA assay. (**a**) Yeast filter lift assay procedure. (*a*) After 3 days incubation, colonies are grown on surface of solid medium. (*b*) Filter paper is fully touched onto the surface of medium, avoid air bubble. Quickly and carefully lift the filter paper with yeast colonies attached onto it. (*c*) Fully immerse the yeast colonies attached paper in liquid nitrogen for 20 s. (*d*) After completely thaw, place the filter paper with colonies facing up on another filter paper presoaked with Z buffer/X-gal solution in the petri dish, avoid air bubble. Incubate the filter paper in 30 °C incubator until colony color changes. (**b**) Schematic diagram of filter lift assay results. CK + is the positive control samples using characterized pair of TALENs, few white color dots are the random case that *lacZ* gene function is not restored. The middle round shaped graph with gray dots is the yeast sample with tested TALEN pair. *Dots* with *gray color* represent the moderate activity comparing with the high activity of positive control (as indicated with *black dots*), several *black dots* dictate the background that the *lacZ* genes were functional somehow not due to the TALEN action. CK shows the negative control when the yeast cell transformed with empty yeast expression vector together with the construct containing the tested binding targets. White dot indicates that the color of yeast colonies do not change at all. No β-galactosidase is expressed. Random black dots are background colonies. (**c**) Quantitative assay results. The tube labeled with CK + and filled with black color in the lower part means high β-galactosidase activity. It usually displays deep yellow color when treated with substrate *ortho*-Nitrophenyl-β-galactoside (ONPG). The one labeled with S and lower part filled with gray color means moderate TALEN nuclease activity that usually turn the colorless substrate into light yellow color. And the CK-tube with transparent color means the target construct and empty yeast expression vectors do not generate background β-galactosidase activity, indicating no color change

3. Synthesize oligonucleotides containing TALEN target sites and two cohesive ends that are compatible with the ones generated by *BglII* and *SpeI*. Mix and boil 50 fmol of each of the oligonucleotide pairs that will form the TALEN target, then allow them to slowly cool down to room temperature.
4. Ligate the annealed oligonucleotides into pCP5. Detailed construction information is as previously described (Umezu et al. 1971).

Qualitative Assay

Assay with Filter Lift

1. Simultaneously transform YPH500 yeast cells with pCP3M containing the left TALEN construct, pCP4M containing the right TALEN construct, and pCP5 containing the TALEN target, and plate on SC-His-Leu-Trp medium.
2. After 3 days of incubation at 30 °C, perform a filter lift assay following the yeast protocols handbook (Clontech).

3. Briefly, place a filter paper over the surface of the medium where the yeast colonies grow, then quickly but carefully lift the filter paper off and completely immerse it into liquid nitrogen for 20 s.
4. Remove and thaw the filter paper, carefully place it, colony side up, in a petri dish containing another filter paper that is presoaked with Z buffer/X-Gal solution.
5. Incubate the filters at 30 °C until the colonies turn blue.
6. Setup the positive control with characterized TALEN constructs, together with their corresponding reporter plasmid, as a standard. Also, include a negative control using empty constructs and the targets for the experimental TALEN constructs.

Note 11: Avoid air bubbles trapped between the two layers of filter paper.

Quantitative Assay

Although a qualitative filter lift assay is easy to perform and saves time, in our experience, it shows a high background and is not precise based on comparisons between TALENs with similar activities. A highly accurate quantitative assay can be performed to measure TALEN activity. The detailed protocols were described before (Townsend et al. 2009), but will be briefly repeated below.

1. Transform pCP3M containing the left TALEN construct and pCP4M containing the right TALEN construct into yeast strain YPH499 and select on SC-His-Leu, and pCP5 containing the TALEN target sequence into strain YPH500, and select on SC-Trp.
2. After several days, pick three single colonies separately and grow them in SC-His-Leu or SC-Trp liquid medium to a cell density of 1.0 at OD600.
3. Mix equal number of yeast cells with effector and target constructs and incubate for at least 6 h in YPAD liquid medium.
4. Centrifuge and wash the cells with SC-His/Leu/Trp selection medium twice.
5. Remove a portion of the mated cells for growth in 2 mL of SC-His-Leu-Trp liquid medium overnight at 30 °C with shaking.
6. Measure the OD600 and determine the β-galactosidase activity with a yeast β-galactosidase assay kit following the manufacturer's instructions.

Yeast Cell Growth Assay for Possible TALEN Toxicity

1. To detect TALEN toxicity, transform YPH500 cells with individual TALEN plasmids or an empty vector as a control. Select transformed cells on SC-His or SC-Leu medium.
2. Choose three single colonies for each plasmid and grow each to a cell concentration of 1.0 at OD600.
3. Serially dilute cells from 1×10^6 to 1×10^3/mL, and apply 10 μL of each as a spot on the surface of SC-His (for pCP3M and its derived plasmids) or SC-Leu (for pCP4M and its derived plasmids) solid medium and allow each to grow for 4 days.
4. Judge toxicity by observing cell viability and proliferation as compared to the control transformations containing the corresponding empty vector.

27.5 Conclusions

Because of the broad range of fungal species studied and a lack of fungal-related engineered nuclease applications in the literature, tailored protocols will need to be developed if TALENs are used for modification of fungal genomes. With that said, TALEN-mediated Genome Editing has proven to be a robust platform and has been broadly adopted by researchers interested in exploiting Genome Editing technology. TALENs have been successfully used in at least 25 organisms from fungus to cultured human cells, with applications ranging from small modifications to larger genome rearrangements. Engineering TALENs is relatively simple, especially considering the many kits that are available through several academic institutions and nonprofit repositories, such as Addgene. This technology will most likely be of great value to researchers interested in fungal applications, such as targeted genome modification or use of

artificial transcription factors for regulation of specific genes.

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