REVIEW

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Transformation of *Saccharomyces cerevisiae* and other fungi

Methods and possible underlying mechanism

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Abbreviations: cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; LiAc, lithium acetate; LiCl, lithium chloride; PEG, polyethylene glycol; WT, wild type; YPD, yeast extract, peptone and dextrose

Transformation (i.e., genetic modification of a cell by the incorporation of exogenous DNA) is indispensable for manipulating fungi. Here, we review the transformation methods for *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Pichia pastoris* and Aspergillus species and discuss some common modifications to improve transformation efficiency. We also present a model of the mechanism underlying *S. cerevisiae* transformation, based on recent reports and the mechanism of transfection in mammalian systems. This model predicts that DNA attaches to the cell wall and enters the cell via endocytotic membrane invagination, although how DNA reaches the nucleus is unknown. Polyethylene glycol is indispensable for successful transformation of intact cells and the attachment of DNA and also possibly acts on the membrane to increase the transformation efficiency. Both lithium acetate and heat shock, which enhance the transformation efficiency of intact cells but not that of spheroplasts, probably help DNA to pass through the cell wall.

Introduction

Transformation is an important technique in which exogenous DNA is introduced into a cell, resulting in genetic modification. In the case of fungi, the spheroplasts of the budding yeast *Saccharomyces cerevisiae* were first successfully transformed in 1978.[1] Several methods to transform intact cells, including the lithium, electroporation, biolistic and glass bead methods, have been developed, and the efficiency of each method has been improved.[2-19] These methods can be used for transforming other fungi such as yeasts (e.g., *Schizosaccharomyces pombe*, *Candida albicans* and *Pichia pastoris*) and filamentous fungi (e.g., Aspergillus species),[17,18,20-34] although the efficiency of transformation of these fungi is generally lower than that of *S. cerevisiae*.

For successful fungal transformation, exogenous DNA must pass through the cell wall and plasma membrane and be delivered in the cytosol to reach the nucleus. During the transformation of spheroplasts, however, exogenous DNA does not need to pass through the cell wall. The mechanism underlying transformation has not been clarified completely even in *S. cerevisiae*, although it has been proposed by several recent studies.[35-38]

In 2001, Gietz and Woods[39] reviewed the methods of fungal transformation and discussed the mechanisms involved, in the context of *S. cerevisiae*. However, several improvements have been achieved since then. It is therefore appropriate to review these methods, especially the mechanism of *S. cerevisiae* transformation, and focus on the recent developments. Here, we review the methods of transformation of *S. cerevisiae*, *S. pombe*, *C. albicans*, *P. pastoris* and Aspergillus species and discuss some common modifications to improve the transformation efficiency. The basic protocols of *S. cerevisiae* transformation are also provided. We also present a model of the mechanism underlying *S. cerevisiae* transformation, based on the recent reports[35-38] and the mechanism underlying transfection in the mammalian system. Although methods requiring special equipments (e.g., electroporation and the biolistic method) are described, we focus on those not requiring special equipments, by concentrating on biological components and events. We have used the term 'transformation efficiency' to mean the number of transformants per microgram of DNA and the term 'transformation frequency' to indicate the transformation efficiency per viable cell number.

Transformation of *S. cerevisiae*

Spheroplast method. Transformation of *S. cerevisiae* by the spheroplast method was first performed by Hinnen et al.[1] They transformed spheroplasts, prepared by enzymatic digestion of *S. cerevisiae leu2 3–112* mutant cells, with chimeric plasmid DNA containing *LEU2* but not a yeast replicon. The transformation

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Table 1. Protocol for the spheroplast method developed by Burgers and Percival[7]

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 (1% yeast extract, 2% bactopeptone, and 2% dextrose) to a concentration of about 3 x 10^7 cells/ml and harvest.
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 them in 20 ml of SCEM [1 M sorbitol, 0.1 M sodium citrate (pH 5.8), 10 mM EDTA and 30 mM 2-mercaptoethanol], add 1,000 U of lyticase, and incubate at 30°C with occasional inversion.
3. After spheroplasting, measure the decrease in the OD_{800} of a 10-fold dilution of spheroplasts in water. Harvest the spheroplasts for 3–4 min when the spheroplasting proceeds to 90% (~15–20 min).
4. Gently resuspend the spheroplasts in 20 ml of 1 M sorbitol by using a 1-ml pipette and pellet for 3–4 min. Then, gently resuspend them in 20 ml of STC [1 M sorbitol, 10 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$] and pellet again for 3–4 min. Resuspend this pellet in 2 ml of STC.
5. Mix aliquots (100 μl) with plasmid DNA and carrier DNA (calf thymus or *E. coli*) added to a total of 5 μg of DNA in <10 μl.
6. After 10 min at room temperature, add 1 ml of PEG [10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and 20% PEG 8000; filter-sterilized], gently resuspend the spheroplasts, and harvest them for 4 min after another 10 min.
7. Resuspend the pellet in 150 μl of SOS (1 M sorbitol, 6.5 mM $CaC1_2$, 0.25% yeast extract and 0.5% bactopeptone; filter-sterilized) and leave at 30°C for 20–40 min. Dilutions of the spheroplasts are made in the same medium.
8. Add 8 ml of TOP [1 M sorbitol and 2.5% agar in selective SD medium (0.67% yeast nitrogen base and 2% glucose)] kept at 45–46°C. Invert the tube quickly several times to mix and plate the suspension immediately on selective SORB plates (SD plates containing 0.9 M sorbitol and 3% glucose).

efficiency was only 30–50 transformants/μg of plasmid DNA, which must integrate into chromosomal DNA for establishing stable transformants. In the same year, Beggs[6] improved the efficiency of this method to 1 x 10^4 transformants/μg of plasmid DNA by using chimeric plasmid DNA carrying an endogenous, autonomously replicating 2 μm yeast element. The highest transformation efficiency—2 x 10^7 and 5 x 10^6 transformants/μg of single-stranded and double-stranded plasmid DNA, respectively—has been achieved by Burgers and Percival.[7] They reduced the number of steps required to prepare spheroplasts, the concentration of cells required at the spheroplasting stage, and the concentration of spheroplasts required during transformation as well as the g forces required for spheroplast pelleting. The transformation frequency was surprisingly high at about 0.10, when the concentration of spheroplasts was 3 x 10^7–3 x 10^8/ml and the amount of single-stranded mPY2 DNA was 1–3 μg. However, the frequency dramatically decreased to 0.0014 when the concentration of spheroplasts was 2 x 10^9/ml and the amount of mPY2 DNA was 16 μg, indicating that the concentration of spheroplasts and amount of DNA are critical for high transformation frequency. When the authors used carrier DNA (calf thymus or *Escherichia coli*), the transformation frequency again depended on the concentration of the spheroplasts and carrier DNA. Transformation

Table 2. Original protocol for the lithium method developed by Ito et al.[2]

1. Grow the yeast cells aerobically on 100 ml of YPD medium at 30°C with reciprocation. At the mid-log phase, harvest the cells by centrifugation, wash once with TE [10 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA] and suspend in TE to a final concentration of 2 x 10^8 cells/ml.
2. To a 0.5-ml portion of this cell suspension, add an equal volume of 0.2 M metal ions (LiAc). After 1 h at 30°C with shaking (140 rpm; stroke, 7.0 cm), incubate 0.1 ml of the cell suspension statically with 15 μl of a plasmid DNA solution (670 μg/ml) at 30°C for 30 min.
3. Add an equal volume of 70% PEG 4000 dissolved in water and sterilized at 120°C for 15 min and mix thoroughly on a vortex mixer. After standing for 1 h at 30°C, incubate the suspension at 42°C for 5 min.
4. Immediately cool the cells to room temperature, wash twice with water, and suspend in 1.0 ml of water.
5. For selecting the yeast transformants, directly spread 0.1 ml of the cell suspension on selective solid medium.

with double-stranded DNA was generally two- or fivefold less efficient than that with single-stranded DNA, but the reason for this difference remains to be elucidated. The protocol for the spheroplast method developed by Burgers and Percival[7] is presented in **Table 1**. It is noteworthy that lithium acetate (LiAc) and heat shock (incubation of spheroplasts at 42°C for a short time in the presence of plasmid DNA) are not required for transformation, as will be discussed later in this review.

Despite the high transformation frequency, the spheroplast method failed to become an important and popular protocol, probably because of the tedious and complex procedure and because the transformants are embedded in regeneration agar, hindering replica plating. This method is still useful for transformation with yeast artificial chromosomes having a length of 100–1,000 kb and with infectious prion particles.[40,41]

Lithium method. During a seminar held on 15 May 1981 at the Research Institute for Food Science (Kyoto University, Japan), the possibility for transforming intact *S. cerevisiae* cells was first presented by one (KM) of the authors of this review, together with other results obtained during his stay at the Mitsubishi-Kasei Institute of Life Sciences (Tokyo, Japan). The lithium method was published in 1983.[2] One of the important findings was that monovalent cations such as Na^+, K^+, Rb^+, Cs^+ and particularly, Li^+ but not divalent cations such as Ca^{2+} (effective for *E. coli* transformation) enhance the transformation efficiency of intact *S. cerevisiae* cells. The reason for the effectiveness of these monovalent cations might be attributed to their mild chaotropic effect during the transformation,[42] and also is discussed later in this review. The original protocol for the lithium method developed by Ito et al.[2] is presented in **Table 2**. LiAc was found to be 1.7-fold more effective than lithium chloride (LiCl). Importantly, lithium was not the sole contributor to the transformation of intact cells. Ito et al.[2] showed that (1) incubation of intact cells with polyethylene glycol (PEG) and plasmid DNA is essential for transformation, (2) short-term incubation of intact cells with PEG and plasmid DNA at 42°C (heat shock) enhances the transformation efficiency and (3) transformation of the cells is most effective at the mid-log phase. The lithium method developed by Ito et al.[2] yielded about 450 transformants/μg of plasmid DNA. PEG was tested according

to its use in the spheroplast method. Lithium was tested because it is known to be effective in eluting inorganic polyphosphate, a negatively charged macromolecule similar to DNA, from anion-exchange columns (Murata K, et al. Agric Biol Chem 1978; 42:2221-6).

By their results, Ito et al.[2] established 4 principles of the lithium method: (1) PEG is essential; (2) LiAc and (3) heat shock enhance the transformation efficiency; and (4) the highest efficiency is obtained when the cells are at the mid-log phase (OD_{610} = 1.6). As this method is faster, simpler and easier to perform than the spheroplast method and needs neither regeneration agar nor special equipment, it has become popular.

Based on these 4 principles, several modifications that enhance the transformation efficiency of the lithium method have been reported. Gietz and co-workers succeeded in improving the efficiency to 5 x 10^6–1 x 10^7/μg of plasmid DNA from 10^8 cells by immediately mixing washed intact cells with PEG, LiAc, plasmid DNA and single-stranded carrier DNA and incubating them at 42°C for 40–60 min without pretreatment.[8-11] The protocol for the modified method (LiAc/single-stranded carrier DNA/PEG method) described by Gietz and Woods[11] is described in **Table 3**. In addition, Gietz and Schiestl[12] stored intact cells as frozen competent cells in 5% glycerol with 10% dimethyl sulphoxide (DMSO). It should be noted that single-stranded carrier DNA is not effective in the spheroplast method, whereas double-stranded DNA is, indicating that the contribution of carrier DNA to the spheroplast method is different from that to the LiAc/single-stranded carrier DNA/PEG method.[7,8] The role of carrier DNA is yet to be elucidated. Intact cells were only poorly transformed with single-stranded DNA in the LiAc/single-stranded carrier DNA/PEG method, although spheroplasts were transformed effectively with this DNA.[7,10]

Other modifications of the lithium method include the addition of 2-mercaptoethanol,[43] dithiothreitol (DTT),[44] DMSO,[45] or ethanol.[46] Although these reagents were effective in the original lithium method, at least 2-mercaptoethanol and DMSO were not effective in the LiAc/single-stranded carrier DNA/PEG method.[10] Chen et al.[13] reported that the addition of 100 mM DTT is effective even in the LiAc/single-stranded carrier DNA/PEG method. Notably, they incubated a mixture of DTT, LiAc, single-stranded carrier DNA, PEG, intact cells and plasmid DNA at 45°C, but not 42°C and stated that incubation at 42°C or 48°C dramatically reduces the efficiency. Another modification is to exclude LiAc.[14,15] Namely, intact cells are transformed with plasmid DNA by incubating the cells with PEG and plasmid DNA at 30°C and then at 42°C (heat shock). The results of this transformation vary according to the strain and the reaction mixture is simple, implying that a biological component is involved.[14,15] As described later in this review, we attempted to clarify the mechanism underlying such transformation.[35,36]

Table 3. Protocol for the LiAc/single-stranded carrier DNA/PEG method developed by Gietz and Woods[11]

1. Inoculate the yeast strain into 5 ml of liquid medium (2x YPAD or synthetic complete [SC] selection medium) and incubate overnight at 30°C. Place a bottle of double-strength YPAD broth (2x YPAD) and 250 ml culture flask in the incubator as well.
2. Determine the titer of the yeast culture by measuring the OD_{600} of a solution of 10 μl of the cells added to 1.0 ml of water in a spectrophotometer cuvette. For many yeast strains, a suspension containing 1 x 10^6 cells/ml will give an OD_{600} of 0.1.
3. Transfer 50 ml of the pre-warmed 2x YPAD to the pre-warmed culture flask and add 2.5 x 10^8 cells to give a density of 5 x 10^6 cells/ml. Incubate the flask on a rotary or reciprocating shaker at 30°C and 200 rpm. (Note: It is important to allow the cells to complete at least 2 divisions. Transformation efficiency remains constant for 3 to 4 cell divisions).
4. When the cell titer is at least 2 x 10^7 cells/ml, which should take about 4 h, harvest the cells by centrifugation, wash the cells in 25 ml of sterile water, and wash again in 1 ml of sterile water.
5. Add water to a final volume of 1.0 ml and vigorous vortex-mixing to resuspend the cells. Pipette 100 μl samples (~10^8 cells) into 1.5-ml microcentrifuge tubes, one for each transformation, centrifuge at top speed for 30 s, and discard the supernatant.
6. Add 360 μl of transformation mix, consisting of 240 μl PEG 3350 [50% (w/v)], 36 μl LiAc (1.0 M), 50 μl boiled single-stranded DNA (2.0 mg/ml), and 34 μl plasmid DNA plus water, to each transformation tube and resuspend the cells by vigorous vortex-mixing.
7. Incubate the tubes in a 42°C water bath for 40 min. [Note: The optimum time can vary for different yeast strains].
8. Microcentrifuge at top speed for 30 s and remove the transformation mix with a micropipette. Pipette 1.0 ml of sterile water into each tube, stir the pellet with a micropipette tip, and vortex.
9. Plate appropriate dilutions of the cell suspension onto SC selection medium.

Electroporation. Electroporation was first used to transform intact *S. cerevisiae* cells by Hashimoto et al.[3] Delorme[16] attempted to establish the optimal conditions for electroporation, by suspending intact *S. cerevisiae* cells in YPD (yeast extract, bactopeptone and dextrose) medium, performing electroporation and plating them directly on selective medium. The optimal conditions were as follows: (1) voltage of 900 V, (2) electroporation of early log-phase cells (OD_{600} = 0.3–1.0) at a cell density of OD_{600} 10–20 in the presence of less than 0.1 μg of plasmid DNA for high transformation efficiency and (3) absence of carrier DNA because it is not effective. Delorme obtained 1,000–4,500 transformants/μg of plasmid DNA. However, Weaver et al.[47] demonstrated that 35–75% of intact *S. pombe* cells (14–25 μl of a solution containing 6 x 10^7 cells/ml) could take up macromolecules (labelled dextran; 70 kDa) within 5 min of a pulse, suggesting that much higher numbers of transformants can be obtained by electroporation. Becker and Guarente[48] attributed the low transformation efficiency of electroporation to inadequate support of electrically compromised cells. They provided continuous osmotic support (with 1 M sorbitol) and routinely obtained 2–5 x 10^5 transformants/μg of plasmid DNA in *S. cerevisiae* transformation. Since then, sorbitol has been included in the standard protocol of the electroporation method of *S. cerevisiae* transformation. Furthermore, Thompson et al.[17] found that the pre-incubation of cells in the presence of both 100 mM LiAc and 10 mM DTT improves the transformation efficiency by an order of magnitude of one to two. LiAc and DTT were synergistically effective. Their method was also applied to the transformation of *C. albicans*.[17] Suga and Hatakeyama[18] reported that the freezing of intact cells of *S. cerevisiae* and *S. pombe* in

Table 4. Protocol for electroporation of frozen competent cells developed by Suga and Hatakeyama[18]

1. Grow *S. pombe* cells in SD medium supplemented with appropriate nutrients to a density of approximately 1 x 10^7 cells/ml at 30°C. Grow *S. cerevisiae* cells in YPD medium to a density of approximately 1 x 10^7 cells/ml at 30°C.
2. Place the cultures on ice for 15 min just before harvesting. Collect the cells by centrifugation and wash the resulting pellet thrice with ice-cold sterilized water. Suspend this pellet in ice-cold freezing buffer containing 0.6–2.5 M sorbitol, 5–10 mM $CaCl_2$ and 10 mM 2-(4-[2-hydroxyethyl]-1-piperazinyl)ethanesulphonic acid (HEPES; pH 7.5) to give a density of approximately 5 x 10^8 cells/ml.
3. Dispense aliquots (0.1 ml) of the cell suspension in 1.5-ml microcentrifuge tubes, slowly freeze them, and store by placing them directly in a -80°C freezer (cooling rate = ~10°C/min).
4. For each electroporation, quickly thaw the frozen competent cells in a water bath at 30°C (warming rate = ~200°C/min) and wash once with 1 ml of ice-cold 1.0 M sorbitol by centrifugation. Resuspend the final pellet in 1.0 M sorbitol to give a density of 1–2 x 10^9 cells/ml.
5. Mix the cell suspension with 0.5–10.0 ng of purified plasmid DNA and then transfer to a chilled cuvette with a 0.2-cm electrode gap. Apply a high electric pulse to the cell suspension, by using the Bio-Rad Gene Pulser II with Pulse Controller Plus.
6. Immediately dilute the electroporated cells in 1 ml of ice-cold 1.0 M sorbitol and spread an aliquot (0.1–0.2 ml) on minimal selection plates. For *S. cerevisiae*, the minimal selection plates contain 1.0 M sorbitol as an osmotic stabilizer.
7. The transformant colonies appear within 4–6 days at 30°C.

sorbitol (0.6–2.5 M) with calcium (5–10 mM) at -80°C results in high transformation efficiency by electroporation, giving more than 10^6 transformants/μg of plasmid DNA after thawing. The protocol for the Suga and Hatakeyama[18] electroporation method of *S. cerevisiae* and *S. pombe* is described in **Table 4**.

Biolistic method. Cells can be transformed with DNA-coated metal microprojectiles that are shot into cells.[49] Armaleo et al.[4] demonstrated that intact *S. cerevisiae* cells can be transformed by the biolistic (bombardment) method. They found that cells at the mid-stationary phase, unlike in the case of the lithium and spheroplast methods, are the most effective. Similar to the spheroplast method, osmotic support with 0.75 M sorbitol and 0.75 M mannitol is needed for high transformation efficiency. Among the metals tested (W, Pt, Fe, Au and Ir), tungsten (W) is the most effective. Stable nuclear transformants result primarily from the penetration of a single particle with a diameter of 0.5–0.65 μm. To coat tungsten, the addition of an appropriate amount of $CaCl_2$ and spermidine to plasmid DNA is important. Optimal conditions, however, yield only about 500 transformants/μg of plasmid DNA from 10^8 cells. Although the transformation efficiency of the biolistic method is low, mitochondrial transformation of *S. cerevisiae* has so far been successful only by this method.[19] Johnston et al.[19] coated 1 μm tungsten with YEp352 (multicopy plasmid bearing wild type [WT] nuclear *URA3*) and pQAoxi3 carrying mitochondrial DNA. The coated tungsten was bombarded on *S. cerevisiae* respiratory-deficient (mit^-) cells having a lesion in nuclear *URA3*. Among the 3,600 Ura^+ nuclear transformants obtained, only six were respiratory-competent and shown to be mitochondrial transformants.

Glass bead method. Constanzo and Fox[5] demonstrated that intact *S. cerevisiae* cells can be transformed by agitation with glass beads in the presence of carrier DNA and plasmid DNA at very low efficiency, namely about 300 transformants/μg of plasmid DNA. Osmotic support (with 1.0 M sorbitol) is required in the selective solid medium.

Transformation of Other Fungi

Transformation of *S. pombe*. The spheroplast method yielded 1 x 10^4–5 x 10^4 transformants/μg of plasmid DNA in *S. pombe*.[20] This method was improved by Allshire[50] to give 5 x 10^5 transformants/μg of plasmid DNA from 4 x 10^7 spheroplasts by using the cationic liposome-forming reagent lipofectin. In this procedure, a linear minichromosome longer than 500 kb was introduced into the *S. pombe* cells. With regard to the lithium method, Okazaki et al.[21] established a protocol that yielded 10^6 transformants/μg of plasmid DNA from 10^8 cells. They found lithium to be the most effective among the tested cations (Li, Na, K, Rb and Cs) and acetate to be better than chloride, as in the case of *S. cerevisiae*.[2] Remarkably, the transformation efficiency sharply depended on the pH of LiAc, with the optimal pH ranging from 4.9 to 5.1. Carrier DNA was not included in the protocol. Morita and Takegawa[51] reported a simplified procedure to give approximately 8,000 transformants/μg of plasmid DNA and they used a colony grown on solid minimal medium. Carrier DNA was included in their protocol, which enhanced the efficiency by about 60-fold. Suga and Hatakeyama[52] established a rapid procedure using cryopreserved competent cells. As a cryoprotectant, they found glycerol (optimal concentration; 30%) to be better than DMSO. Glycerol also enhanced the transformation efficiency. This procedure yielded more than 10^6 transformants/μg of plasmid DNA. Electroporation could be used to transform *S. pombe* cells and yielded 10^4–10^6 transformants/μg of plasmid DNA (**Table 4**).[18]

Transformation of other yeasts. *P. pastoris* cells have been transformed at 10^5 transformants/μg of plasmid DNA by the spheroplast method and at 4 x 10^6 transformants/μg of plasmid DNA by electroporation.[22,23] In the electroporation method, *P. pastoris* cells are pretreated with 100 mM LiAc and 10 mM DTT, as in the case of *S. cerevisiae*.[17] This pretreatment increases the transformation efficiency of *P. pastoris* and *S. cerevisiae* cells by approximately 150- and 6–300-fold, respectively. The lithium method is also applicable to *P. pastoris* (protocol available at http://www.invitrogen.com/). LiCl, but not LiAc, is effective but the efficiency is very low, namely about 17 transformants/μg of plasmid DNA.

The spheroplast method has been used to transform *C. albicans* cells, yielding 0.5–5 transformants/μg of plasmid DNA.[24] This method has been improved to yield 10^3–10^4 transformants/μg of plasmid DNA.[25] The lithium method yielded only 50–100 transformants/μg of plasmid DNA.[26] The protocol for the lithium method of *C. albicans* transformation has been described in detail by Ramon and Fonzi.[53] With regard to the electroporation method, pretreatment of the cells with 100 mM LiAc and 10 mM DTT enhances the efficiency by 3.5-fold and

yields approximately 160 transformants/μg of plasmid DNA.[17] De Backer et al.[27] optimized the conditions and improved the efficiency to yield 4,500 transformants/μg of plasmid DNA. They reduced the pretreatment concentration of LiAc from 100 mM to 5 mM, used a lower amount of plasmid DNA (0.1 μg) and immediately plated the pulsed cells on selective solid medium containing 1 M sorbitol.

Transformation of Aspergillus species. The spheroplast method established for *S. cerevisiae* transformation was adapted to *Aspergillus nidulans* by Tilburn et al.[28] in 1983. They achieved an efficiency of only 25 transformants/μg of plasmid DNA. More recently, Dawe et al.[29] improved the efficiency to several hundred transformants per microgram of plasmid DNA. In brief, conidiospores were germinated and their cell walls were enzymatically digested.[29,30] The protoplasts were incubated with plasmid DNA, PEG and $CaCl_2$ and spread on selective solid medium. On this medium, heterokaryotic transformants were obtained. As conidia are generally uninucleate, homokaryotic transformants can be readily obtained from heterokaryotic ones by reselecting the progeny. The protocol for *Aspergillus oryzae* transformation by the spheroplast method has been described in detail by Kitamoto.[31] Transformation of *A. nidulans* by electroporation and the biolistic method has been reported by Sanchez and Aguirre[32] and Barcellos and Fungaro,[33] respectively. Gouka et al.[34] described the transformation of *Aspergillus awamori* by using *Agrobacterium tumefaciens*. By their method, it is possible to construct recombinant mold strains free from bacterial and other foreign DNA and this system is expected to stimulate the market acceptance of fungus-derived products by avoiding the introduction of bacterial and other foreign DNA into fungi.

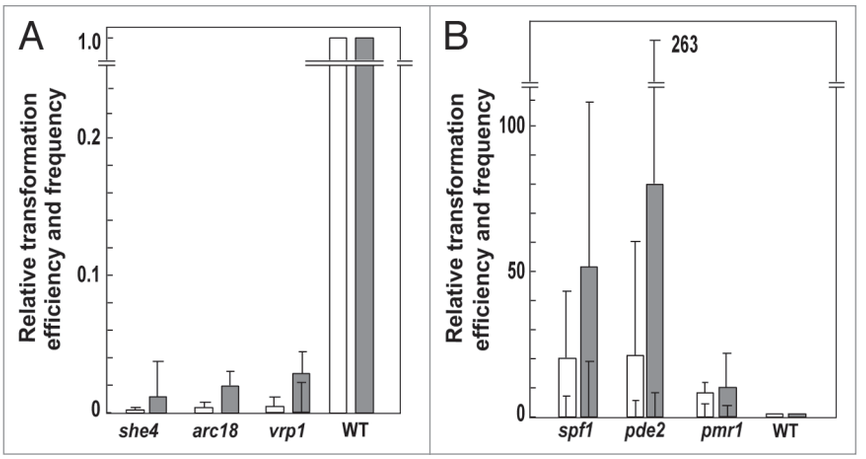

Figure 1. Relative transformation efficiency (white bar) and frequency (grey bar) of the low-transformability mutants (A) and high-transformability mutants (B).[35] The values are relative to those of the WT (BY4742), set at 1.0. The averages and minimum or maximum of 3 independent experiments are shown.

Molecular Mechanism Underlying *S. cerevisiae* Transformation

Endocytosis. *S. cerevisiae* cells can be transformed only by incubating the cells with DNA and PEG.[14,15] The transformation efficiency depends on the strain background and the composition of the reaction mixture is simple, suggesting that biological components and events are involved in this type of transformation.[14,15] In order to elucidate the underlying mechanism, we used this method to determine the transformation efficiency and frequency of approximately 5,000 strains in which each of the nonessential genes is deleted.[35] Endocytosis is a vesicular transport pathway used by eukaryotic cells to internalize plasma membrane molecules, extracellular fluid and particles.[54-57] As described below, we obtained evidence that DNA enters the cell via endocytotic membrane invagination.

Our comprehensive search for transformability initially identified *she4* and *arc18* as low-transformability mutants. We use the term 'transformability' to indicate both the transformation efficiency and the transformation frequency. Several other low-transformability mutants were also identified, including *vrp1*, *pan1-9*, *pan1-20* and *las17*,[35] as well as *myo3myo5*, *arp2*, *arp3*, *arc15* and *arc19* (our unpublished data). The very low transformability of *she4*, *arc18* and *vrp1* is shown in **Figure 1A**. The Arp2/3 complex consists of 7 subunits (Arp2, Arp3, Arc35, Arc19, Arc18, Arc15 and Arc40), each of which contributes differently to the assembly and function of the complex.[58] The activation of the Arp2/3 complex requires Myo3/5, Vrp1, Las17 and Pan1.[54,55,59] She4 is a molecular chaperone needed for the function of Myo3/5.[60] Hence, all of these low-transformability mutants lack a component of the Arp2/3 complex or a component required for the activation of this complex. The Arp2/3 complex and its activation are necessary for the endocytotic uptake accompanying membrane invagination.[54-57] Furthermore, increasing evidence confirms that cationic lipid-DNA complexes (lipoplexes) and cationic polymer-DNA complex (polyplexes) enter mammalian cells via endocytosis.[61-66] Considering all these reports, we propose that during the transformation of *S. cerevisiae*, exogenous DNA enters the cell via endocytosis-like membrane invagination.[35] However, it should be noted that other endocytotic mutants such as *sac6*, *end3*, *rvs161*, *rvs167*, *akr1*, *erg2*, *sla1*, *kcs1* and *arg82* showed normal transformability.[35] The reason for this remains unknown.

In the case of mammalian cells, the term 'transfection' is used instead of transformation. Gene transfer mediated by lipoplexes and polyplexes is useful for cellular transfection, potentially for non-viral gene therapy and involves endocytosis.[61-66] Rejman et al.[63] concluded that lipoplexes enter cells via clathrin-mediated endocytosis, whereas polyplexes are taken up by 2 endocytotic routes, one involving caveolae and the other involving clathrin-coated pits. Hufnagel et al.[66] provided evidence that the uptake of polyplexes also involves fluid-phase endocytosis which is considered to be one of the macropinocytotic pathways. Only clathrin-dependent endocytosis is known in *S. cerevisiae*.[57]

Enhancement of transformability by altered cell wall structure. We identified *pde2*, *pmr1* and *spf1* as high-transformability

mutants in *S. cerevisiae* (**Fig. 1B**).[35] The *spf1* mutant exhibited the highest transformability (**Fig. 1B**). In *pde2*, which lacks high-affinity cyclic adenosine monophosphate (cAMP) phosphodiesterase, the basal cAMP is elevated. On the other hand, the *pde1* mutant, lacking low-affinity cAMP phosphodiesterase (where the cAMP level is not affected), showed normal transformability. Therefore, we predict that elevated basal cAMP enhances transformability. Although cAMP-dependent protein kinase (PKA) is a major cAMP target in the Ras-cAMP pathway,[67] we failed to determine the target of PKA because we found *msn2, msn4, rim15* and *sok2*, in which known transcription factors controlled by PKA are disrupted, have normal transformability.[67] Tomlin et al.[68] reported that *pde2* exhibits high transformability and sensitivity against hypotonic shock. These 2 characteristics are also observed in the sorbitol-dependent mutant, *srb*, which has a defect in the integrity of the cell wall.[68] In addition, *PDE2* is a multicopy suppressor of the sorbitol dependency of the *srb* mutant. Thus, Tomlin et al.[68] suggested that the Ras/cAMP pathway may modulate cell-wall construction. We therefore propose that the high transformability of *pde2* is attributable to the altered cell wall structure.

Spf1, an endoplasmic reticulum (ER)-located P-type ATPase, is involved in Ca^{2+} homeostasis in the ER. Pmr1 is a Golgi-located Mn^{2+}/Ca^{2+} P-type ATPase. The disruption of *SPF1* or *PMR1* thus alters the ER-Golgi functions, resulting in a wide variety of phenotypes, including those with altered cell wall structure.[69-71] We found that the transformability of *pde2spf1*, *pde2pmr1*, *spf-1pmr1* and *pde2pmr1spf1* is not synergistically higher than that of *pde2*, *pmr1* and *spf1*, indicating that the effects of each deletion of *PDE2*, *PMR1* and *SPF1* are not additive (our unpublished data). These data suggest that the deletion of *PDE2*, *PMR1* or *SPF1* has the same effect on cells to enhance transformability. We propose that, at least in the case of *spf1*, the altered cell wall structure, which can absorb a large amount of DNA, contributes to the high transformability, as described in the next section.

Visualization of the transformation process. YOYO-1 is a widely used cell-impermeable fluorescent DNA probe.[72] Intercalation of YOYO-1 into double-stranded DNA increases its fluorescent intensity by more than 1,000-fold.[73] By using this approach, Zheng et al.[37] observed that YOYO-1-labelled plasmid DNA (pUC18) (YOYO-1/pUC18) attaches to the region around intact cells incubated with PEG, single-stranded carrier DNA, LiAc and YOYO-1/pUC18 at 30°C for 30 min and then at 42°C for 15 min. This finding is in agreement with the observation by Gietz et al.[10] who reported that PEG deposits radio-labelled plasmid DNA onto the cell surface. Furthermore, after washing the cells, Zheng et al.[37] observed some dot-like fluorescent signals, which were considered to be DNA-binding sites. Chen et al.[38] observed that YOYO-1/pUC18 attaches to the region around spheroplasts. We also observed the same behaviour of YOYO-1/YEp13 after incubating intact cells with PEG and YOYO-1/YEp13, which can transform *S. cerevisiae*.[74] Moreover, we confirmed that PEG is indispensable for the attachment of YOYO-1/YEp13 onto cells and their successful transformation,[74] as demonstrated by Chen et al.[38] Using YOYO-1 and flow cytometry, the incubation of intact cells with LiAc or PEG at 30°C or the treatment of the cells at 42°C (heat shock) was found to increase the permeability of the intact cells to YOYO-1.[37]

Because of the low resolution of fluorescence microscopy, no information was obtained regarding the entry of DNA into cells and the delivery of DNA into the nucleus. However, taken together with our proposition that DNA enters cells via endocytotic membrane invagination, we believe that DNA attached to the cell surface must successfully pass through the cell wall before it enters cells via membrane invagination. Correspondingly, we confirmed that washing the cells to remove YOYO-1/YEp13 from the cell surface reduces the transformation efficiency.[74] Furthermore, we found that (1) the cells of the high-transformability mutant *spf1* absorb much more YOYO-1/YEp13 on their cell surfaces than WT cells and (2) the spheroplasts of *spf1* show normal transformability, strongly suggesting that the altered cell wall structure of *spf1* enhances the transformability.[74] This means that the DNA attached to the cell wall, but not the DNA in solution, passes through the cell wall and enters into the cell during transformation. Intact cells with DNA around their cell walls are usually spread on selective solid medium during the transformation procedure. Thus, it is possible that the entry of DNA into the cells and the delivery of DNA into the nucleus mainly occur in cells spread on the selective solid medium.

Transcriptional analysis of the role of PEG in the transformation of intact cells. We found that the pre-incubation of intact *S. cerevisiae* cells with PEG enhances the efficiency and frequency of transformation which can be achieved only by incubating the cells with PEG and DNA, suggesting that PEG may cause an intracellular response.[36] To understand this response, microarray and metabolome analyses were conducted.[36] Microarray analysis revealed that the incubation of the cells without PEG causes the upregulation of several genes, including those involved in carbon-source metabolism (e.g., fatty acid metabolism yielding acetyl-CoA) and those involved in stress responses. Contrary to this finding, the incubation of cells with PEG did not result in any transcriptional change. These microarray results are supported by the results of the metabolome analysis for anionic metabolites, implying that the physical effect of PEG on the cell membrane, rather than the effect of PEG itself on the intracellular response, results in the high efficiency and frequency of transformation. Zheng et al.[37] reported that incubation of intact cells with PEG increases their permeability to YOYO-1. This effect may explain the enhanced efficiency and frequency of transformation that we observed.[36]

Effects of LiAc, heat shock, and PEG on the transformation of intact cells and spheroplasts. We compared the effects of LiAc, heat shock and PEG on the transformation efficiency or frequency of intact cells and spheroplasts (**Table 5**). LiAc and heat shock are required to enhance the transformation of intact cells, but give no effect on transformation of spheroplasts,[2,15,38] implying that LiAc and heat shock help DNA to pass through the cell wall. This is congruent with the observation by Zheng et al.[37] that LiAc and heat shock increase the permeability of intact cells to YOYO-1.

PEG is essential for the transformation of intact cells. It is also indispensable for the attachment of DNA around intact cells and

Table 5. Effects of LiAc, heat shock and PEG on the transformation of intact cells and spheroplasts

	Intact cells	Spheroplasts
LiAc	Enhances the transformation efficiency and frequency (although not indispensable).[2,38]	No effect on the transformation frequency.[38]
	Increases the permeability of intact cells.[37]	
Heat shock	Enhances the transformation efficiency (although not indispensable).[2,15]	No effect on the transformation efficiency.[74]
	Increases the permeability of intact cells.[37]	
PEG	Indispensable for transformation efficiency.[2,15]	Not indispensable for transformation frequency but enhances the frequency.[38]
	Pre-incubation enhances the transformation efficiency and frequency.[36]	
	Increases the permeability of intact cells.[37]	
	Indispensable for DNA attachment.[38]	Indispensable for DNA attachment.[38]

spheroplasts.[2,15,38] It should be noted that PEG is not indispensable for the transformation of spheroplasts but enhances their transformation frequency.[38] In the case of intact cells, the attachment of DNA around the cell wall is probably indispensable for transformation. Several reports suggest that the attached DNA, but not the DNA in solution, passes through the cell wall and enters the cell via endocytotic membrane invagination (under submission).[35,37,74] In spheroplasts, the DNA in solution may be able to enter the cells without attaching around the spheroplasts in the absence of PEG. The attachment of DNA around spheroplasts may increase the possibility that DNA enters the cell via endocytotic membrane invagination.

The possibility also remains that PEG directly acts on the membrane to enhance the transformation frequency of spheroplasts,[38] increase the permeability of intact cells,[37] and enhance their transformation efficiency and frequency.[36] As described above, we revealed that PEG caused no intracellular response.[36] Furthermore, PEG could dehydrate the membrane,[75] raise the gel-to-fluid phase transition temperature of the membrane,[76] decrease the fluidity of the membrane,[77] and enhance the permeability of the membrane to Ca^{2+} and other ions.[78]

Conclusion and Perspective

We have presented a model of the mechanism of transformation of intact *S. cerevisiae* cells in **Figure 2**, although this mechanism is not convincingly evidenced. In this model, we propose that (1) DNA initially attaches to the cell wall, (2) passes through the cell wall and (3) enters into the cells via endocytotic membrane invagination. PEG is essential for the attachment of DNA. LiAc and heat shock help the DNA to pass through the cell wall. PEG also possibly acts on the membrane to increase the transformation frequency and efficiency of both intact cells and spheroplasts. The role of carrier DNA has not yet been elucidated.

After DNA enters the cell via membrane invagination, the DNA in the endosomes is delivered to the vacuoles. Hence, this DNA has to escape digestion for successful transformation. Moreover, it has to enter the nucleus. However, nuclear pores permit only the transport of molecules with size less than 70 kDa or a diameter less than 10 nm, which is much smaller than the size of DNA.[79] In the case of the mammalian system, the escape of DNA from the endosomes (endosomal escape) is regarded to be significant for transfection and several models of endosomal escape have been proposed.[61] Further, in the mammalian system, it has been proposed that DNA enters the nucleus during mitosis or through the fusion of lipoplexes with the nuclear membrane.[61] In the case of transformation of *S. cerevisiae*, no information is available on how DNA reaches and enters the nucleus after it enters the cells. Further research is required to understand it and confirm the correctness of our model (**Fig. 2**). In particular, visualization of the transformation process of *S. cerevisiae* by using both electron microscopy and labelled DNA is essential. Moreover, quantitative analysis

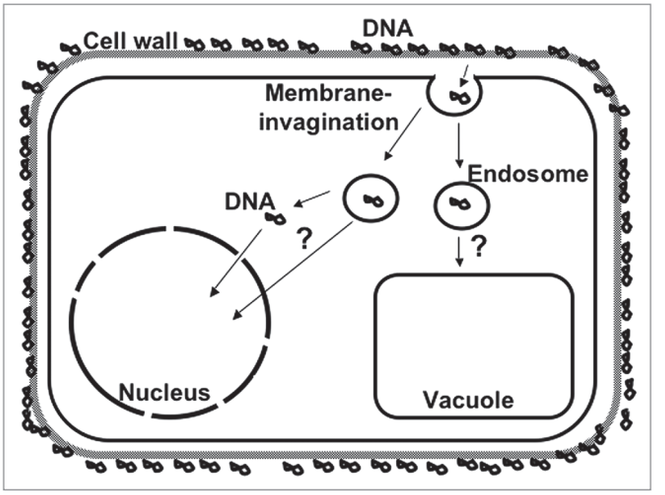

Figure 2. Putative mechanism of *S. cerevisiae* transformation. DNA initially attaches to the cell wall. PEG is indispensable for this attachment and for successful transformation of intact cells. PEG also possibly acts on the membrane to increase the transformation frequency and efficiency as well as the permeability to YOYO-1. The attached DNA passes through the cell wall. LiAc and heat shock help DNA to pass through the cell wall. DNA then enters into the cell via endocytotic membrane invagination. Some DNA in the endosomes is delivered to the vacuoles and digested. However, the manner in which DNA escapes digestion, reaches the nucleus and enters it through the nuclear pore is still unclear.

of the transformation of *S. cerevisiae* is required. For example, in mammalian cells, successful transfection can be quantitatively evaluated by fluorescent-activated cell sorting or by the expression of a reporter gene.[63] In contrast, successful transformation of *S. cerevisiae* can be monitored only by counting the number of transformants and viable cells, which requires 3–4 days and is less quantitative than the methods used for the mammalian system.

Transformation of *S. cerevisiae* and other fungi is indispensable for manipulating these organisms. However, the mechanism underlying the transformation is still unknown. *S. cerevisiae* has many advantages as a model organism and can be used to understand the transformation mechanism. Elucidation of the mechanism will contribute to increasing the transformation efficiency of fungi and clarifying the mechanism underlying mammalian transfection.

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