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Comparison between polyethylene glycol (PEG)- and polyethylenimine (PEI)-mediated transformation of *Aspergillus nidulans*

Authors

Tetsuya Kuwano • Chikako Shirataki • Yasuo Itoh

Affiliations and addresses

T. Kuwano • C. Shirataki

Department of Biological Sciences, Faculty of Science, Shinshu University, Matsumoto, Nagano 390-8621, Japan

Y. Itoh

Graduate School of Shinshu University, Division of Science and Technology, Matsumoto, Nagano 390-8621, Japan

*Present address:*
T. Kuwano
Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan.

Correspondence

Yasuo Itoh

E-mail: ysoitoh@shinshu-u.ac.jp

**Abstract** Genetic transformation of many filamentous fungi is carried out by a protocol that utilizes polyethylene glycol (PEG) and calcium ion ($Ca^{2+}$). This method has remained practically unchanged for more than 20 years, but the roles these molecules play are not definitively understood. To gain a better understanding, we have compared PEG transformation to a protocol using polyethylenimine (PEI) that is the basis for non-viral transfection in mammals and which has a well established molecular model for assisting DNA uptake. Protoplasts of *Aspergillus nidulans* could be transformed in the presence of $Ca^{2+}$ with a relatively high ratio of PEI to DNA molecules. By comparing PEI and PEG in terms of interaction with DNA, fungal protoplasts, and response to different transformation conditions, we propose that the role of PEG is most likely to function after transforming DNA is incorporated into protoplasts, rather than the accepted view that it functions outside of the cell. Confirmation that protoplast fusion was not involved in DNA uptake is consistent with this hypothesis.

**Keywords** Filamentous fungi • Transformation • polyethylene glycol • polyethylenimine • $Ca^{2+}$

## Introduction

Passage of extracellular DNA through the plasma membrane is the first step of genetic transformation, a process that is fundamental to basic studies in biology as well as for technical applications such as gene therapy. Endocytosis is shown to be the major mechanism by which transfer of DNA into a cell occurs in mammals (Khalil et al. 2006), and in *Saccharomyces cerevisiae* (Neukamm et al. 2002; Kawai et al. 2004), but in filamentous fungi, the precise molecular mechanism for this process has yet to be revealed.

A procedure for obtaining stable transformants by chromosomal integration of extracellular DNA in a model fungus, *Neurospora crassa*, was first established utilizing calcium ions ($Ca^{2+}$) and polyethylene glycol (PEG) thirty years ago (Hinnen et al. 1978; Case et al. 1979). Another model fungus, *Aspergillus nidulans*, was transformed by the same procedure (Ballance et al. 1983; Yelton et al. 1984). Since then a variety of methods have been developed, which range from electroporation (Chakraborty and Kapoor 1990) to Agrobacterium-mediated procedures (Bundock et al. 1995; de Groot et

al. 1998). Nevertheless the $Ca^{2+}$ and PEG method is still widely used due to its technical convenience and reproducibility (Olmedo-Monfil et al. 2004). Other molecules included in this method are an osmotic stabilizer and pH adjusting buffer, both of which have roles in maintaining protoplasts, rather than directly aiding DNA uptake. Osmotic conditions appear to have a significant role in destabilizing endosomes after extracellular DNA uptake (Neukamm et al. 2002) but are not a prerequisite for transformation to occur.

On the other hand, the roles for $Ca^{2+}$ and PEG in transformation are obscure. $Ca^{2+}$ plays a universal role in a variety of cellular responses, especially in signaling pathways (reviewed by Berridge et al. 2003). Concentrations of $Ca^{2+}$ commonly used in fungal transformation range from 10 to 50 mM (Fincham 1989; Ruiz-Diez 2002) which coincide with the concentration of external $Ca^{2+}$ that causes transient increases of cytosolic $Ca^{2+}$ in filamentous fungi (Nelson et al. 2004). However, none of the pathways that are regulated by $Ca^{2+}$-mediated signaling have been directly connected to the DNA uptake process so far.

PEG is known to cause fusion of protoplasts in filamentous fungi (Anne and Peberdy 1975) and of lipid-bilayers in general (Lentz 1994). Hyphal cells and/or germlings that are used to prepare protoplasts of *A. nidulans* contain multiple nuclei (Fiddy and Trinci 1976), therefore problems associated with the number of nuclei in a cell after protoplast fusion would not be a significant issue for this organism. Therefore, together with controversy regarding the occurrence of endocytosis in filamentous fungi (de Vries et al. 2003), fusion could have been considered as a mechanism for extracellular DNA uptake in filamentous fungi (Koukaki et al. 2003). However, in *S. cerevisiae*, fusion of spheroplasts was shown not to be involved (Burgers and Percival 1987). Furthermore PEG is reported to deposit extracellular DNA onto intact *S. cerevisiae* cells during transformation (Gietz et al. 1995). The volume-exclusion effect of PEG is the basis of this idea (Louie and Serwer 1994), but it has not been shown if this effect can overcome the electrostatic repulsion between DNA and the plasma membrane under conditions used for transformation.

The aim of this paper was to gain a better understanding of the processes involved in transformation of filamentous fungi, particularly in relation to the roles of PEG and $Ca^{2+}$. Firstly, we examined if uptake of extracellular DNA occurs by fusion of

protoplasts in *A. nidulans* and clearly show that these are independent events. Secondly, we compared the conditions for polyethylenimine (PEI)-mediated transformation to that of PEG and show that the proposed critical role of PEG in transformation of *A. nidulans* is unlikely to concern the interaction between extracellular DNA and the cell surface.

## Materials and methods

### Fungal strains and media

*A. nidulans*: A89 (*biA1;argB2*), A272 (*yA2;pyroA4;methB3*), and A773 (*pyrG89;wA3;pyroA4*) were obtained from the Fungal Genetics Stock Center (FGSC). DC25 (*argB2*) was selected from the progeny of a cross between A89 and A773. *Aspergillus* minimal medium (MM) was prepared as described (Pontecorvo et al. 1953; Kafer et al. 1982). MM was supplemented to support the growth of strains as follows: arginine and methionine 7 mM, biotin 0.1 μM, pyridoxin 2.5 μM, uridine and uracil 2.5 and 5 mM, respectively. Sucrose was added at 0.8 M in the media as an osmotic stabilizer for supporting the regeneration of protoplasts after transformation reactions.

### Standard transformation protocols and protoplast fusion

Protoplasts of *A. nidulans* were prepared from hyphal cells obtained by over-night liquid culture in MM as described in Natsume et al. (2004), modified from Yelton et al. (1984). For cell wall digestion, Yatalase (Takara) and Kitalase (Wako) were used at 20 and 5 mg/ml, respectively. After 6 h incubation at 30$^{o}$C with constant shaking (60-80 rpm), hyphal debris was removed by filtration through a single layer of Miracloth (Calbiochem). ST1 buffer (1 M sorbitol, 100 mM Tris-Cl, pH 8.0) was overlaid on the top of filtrate and protoplasts were collected at the interface by centrifugation at 700 x g at room temperature. This protoplast fraction was washed by adding a 5-fold volume of ST2 buffer (1 M sorbitol, 50 mM Tris-Cl, pH 8.0) and centrifuging at 600 x g. Collected protoplasts were further washed with 5 ml of ST2 buffer. Protoplasts were finally suspended in ST2 buffer.

A standard protocol for PEG-mediated transformation was as follows. Protoplast

concentration was adjusted to 2.5 x $10^8$ /ml in ST2 and 40 μl of this suspension ($10^7$ protoplasts) was combined with an equal volume of ST2 containing appropriate concentration of factors to be examined (i.e. cations and osmotic stabilizers). Transforming DNA (0.1 pmol in 5 μl) was added together with 20 μl of PEG buffer [40% w/v (weight/volume) PEG 4000, 50 mM Tris-Cl, pH 8.0, and appropriate concentrations of factors to be examined] followed by incubation on ice for 30 min. This reaction mixture was transferred to 37°C after adding a further 900 μl of PEG buffer, incubated for 10 min and plated onto appropriately prepared media.

For PEI-mediated transformation, the concentration of protoplasts was adjusted to 2.0 x $10^8$ /ml in ST2 and 50 μl of this suspension ($10^7$ protoplasts) was combined with 50 μl of ST2 containing 0.1 pmol transforming DNA, appropriate amount of factors to be examined and PEI (0.8 kDa, Cat. No. 408719, Sigma Aldrich; 2 kDa, Cat. No. 408700, Sigma Aldrich; 70 kDa, Cat. No. 15315-85, Nacalai). The amount of PEI added for a transformation reaction is expressed as PEI nitrogen per DNA phosphate (N/P) ratio, rather than percent, for convenience of comparison with previously published data. Since 0.1 pmol of transforming plasmid pTN51 (10.1 kb) contains 2 nmol of phosphates, PEI that gives N/P ratio of $10^3$ should contain 2 μmol of effective nitrogen. Molecular weight of a unit structure of PEI is 42 and it contains one amine nitrogen (Boussif et al. 1995), therefore 84 μg of PEI is included in 100 μl of transformation reaction. This equals to 0.08% w/v and about one five-hundredth of the amount PEG that was used (36.8% w/v). A commercial solution of PEI 2 kDa is 50% w/v, therefore 420 μl of the original solution was mixed into 100 ml to make 1.25 x ST2, the pH was adjusted to 6.5 with HCl and filter sterilized. One-fifth of the volume of DNA in either sterilized deionized water or TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) was added. Equal volumes of this PEI/DNA solution in ST2 and protoplast suspension were combined. Reaction mixtures were incubated on ice for 30 min followed by 10 min at 37°C, and then plated. The step-wise manner of addition that is used for PEG transformation was not performed for PEI.

When efficiency of protoplast fusion was examined, protoplasts of two strains having different marker mutations were prepared independently. Concentrations were adjusted to 2.5 x $10^8$ /ml, 20 μl of suspensions were combined to make 40 μl, and the same procedure was carried out as for PEG-mediated transformation. For this

experiment, plates were prepared to distinguish protoplast regeneration (MM supplemented with pyridoxin, methionine, and pyrimidine), fusion of two mutant strains (MM with pyridoxin), transformation of each mutant (MM with pyridoxin, pyrithiamine, and methionine for A272 and pyrimidine for A773, respectively) and transformation accompanied with fusion (MM with pyridoxin and pyrithiamine). Colonies of each mutant strain were recognized by their own conidial color as well; yellow for A272 and white for A773. Transformation/fusion frequency is expressed as number of transformants or colonies by fusion per $10^5$ protoplasts.

Plasmid construction and purification

Media for routine growth and maintenance of *Escherichia coli* were as in Sambrook and Russell (2001). Plasmid DNA was isolated using a Quantum Prep$^{TM}$ Plasmid Midiprep Kit (Bio-Rad). Plasmid pPTRI was purchased from Takara and pDHG25 was obtained from FGSC. Plasmid pTN51 (10.1 kb) was constructed by inserting a 5.3-kb *Hin*dIII fragment from pDHG25 that harbored an AMA1 sequence, into the *Hin*dIII site of pPTRI.

Detection of vector DNA that interacts with protoplasts, PEG, PEI and $Ca^{2+}$

To examine the interaction between PEG and DNA, pBluescript II KS + was incubated in the transformation reaction mixture (0.1 pmol in 25 μl) with different concentrations of PEG and $Ca^{2+}$ but without protoplasts, and was electrophoresed on an agarose gel. Mobility shift in the absence of PEG was used as a control. For detecting the interaction between the transforming DNA (pTN51) and protoplasts, the number of protoplasts in the transformation mixture was increased 2-fold compared to the standard protocol to improve the recovery of nucleic acids. The transformation mixture was then diluted 10-fold with ST2 and centrifuged at 1500 x *g* for 15 min. The resulting pellet was suspended in 1 ml of ST2 and centrifuged again at 1500 x *g* for 15 min. The pellet was further washed with 200 μl of ST2 and finally suspended in 200 μl of ST2. Recovery of protoplasts by centrifugation in the presence of PEG was too poor for nucleic acid extraction; therefore, 2 mM of $Ca^{2+}$ was added to ST2 at the washing step. Collected

protoplasts were treated with Proteinase K (2.5 mg/ml, Nacalai) and RNase A (0.25 mg/ml, Nacalai), and nucleic acids were purified by phenol-chloroform extraction. These samples were run on agarose-gel and chromosomal DNA was quantified with Densitograph (Atto) and used for normalizing nucleic acid concentrations. A 2.2-kb fragment that spanned *ptrA* was amplified with primers TN0229: 5'-GGTGCCCGGGCAACCAAGTCATTCT-3' and TN0230: 5'-TTTACCCGGGCATGTGGATTACGAG-3'. Conditions for PCR are as follows: 2 min at 95$^{o}$C, followed by 30 cycles of 30 sec at 95$^{o}$C, 30 sec at 60$^{o}$C, and 60 sec at 72$^{o}$C, and final extension for 5 min at 72$^{o}$C. Purified pTN51 was used as template for reactions, and a regression line with a reliable correlation coefficient between template concentration and signal intensity was obtained. Signal intensities of PCR products from extraction samples were determined by Densitograph, as long as they were in the reliable range of the standard curve generated from pTN51.

## Results

### Protoplast fusion and DNA uptake

Because PEG is known to cause fusion of fungal protoplasts (Anne and Peberdy 1975; van Solingen and van der Plaat 1977), its possible role in transformation was examined. Protoplasts were prepared separately from two *A. nidulans* mutants (A272 and A773) that had different nutrient-requirements and conidial-color mutations. These were combined and the standard transformation procedure with PEG and $Ca^{2+}$ was performed with pTN51 that had a marker gene for selection by pyrithiamine resistance, followed by plating onto appropriate media to distinguish events.

Fewer transformants were consistently obtained from A773 compared to A272 but the reason for this difference was not further examined. The frequency of transformation and fusion were comparable, but they occurred rarely in the same protoplasts. The number of colonies obtained on an MM plate supplemented with pyridoxine and pyrithiamine (inter-strain fusion and DNA uptake) was about 3% of that was obtained on MM with pyridoxine (inter-strain fusion) at 200 mM of $Ca^{2+}$ (Table 1). Appearance

of colonies was different among these events; colonies resulted by fusion showed sparse mycelial growth from dense-core region and were usually not circle. Although regeneration of protoplasts and frequencies of fusion and transformation were more efficient at 200 mM of $Ca^{2+}$, similar results were obtained at 2 and 20 mM. A difference was observed in response to $Ca^{2+}$ concentration between fusion and DNA uptake, 2 to 20 mM $Ca^{2+}$ was more effective on inter-strain fusion event.

Establishment of PEI-mediated transformation in *A. nidulans*

To obtain an insight into a role of PEG by comparing the well-established mode of action of PEI, conditions were explored for PEI-mediated transformation in *A. nidulans* in terms of molecular weight and amount (Fig. 1). The highest transformation frequency was obtained when 2 kDa PEI was added at N/P ratio of $10^3$. Taking the viability into account, about 1 transformant was obtained from $10^3$ viable protoplasts. This transformation efficiency (number of transformants per viable protoplasts) was comparable to PEG-mediated procedure at 20 mM of $Ca^{2+}$ (Table 1 and Fig. 4**A**). Therefore, considering the deviation in the transformation frequency from different protoplast preparations, PEI could be an alternative mediator for *A. nidulans* transformation.

A correlation between molecular weight and cytotoxicity was observed that has also been reported for mammals (Shin et al. 2005). Also, concentration and cytotoxicity was correlated in 0.8, 2 and 70 kDa of PEI. Cytotoxicity of 0.8 kDa PEI was the least evident but this molecular weight of PEI was not effective for transformation. When $Ca^{2+}$ was not added to the transformation in *A. nidulans*, the protoplasts could not regenerate with 2 kDa PEI at N/P $10^3$, and at N/P $10^2$ regeneration was 5%, about one-fifth of that obtained with 20 mM $Ca^{2+}$. Protoplast regeneration recovered to 20 to 25% without $Ca^{2+}$ at N/P $10^1$, but at both N/P $10^2$ and $10^1$ transformants were hardly ever obtained, indicating that $Ca^{2+}$ was required for PEI-mediated transformation of *A. nidulans*.

Comparison of PEI- and PEG-mediated transformation in *A. nidulans*

The high cationic charge density of PEI mediates the interaction with polyanionic DNA (Boussif et al. 1995). This is illustrated by ethidium bromide-stained gels that show retardation of DNA mobility in response to PEI concentration (shown in Fig. 2A as a control). Conversely, retardation in mobility in response to 32% PEG that was within the concentration range (8 to 36.8%) used for transformation was negligible (Fig. 2B). A mobility shift was caused by 200 mM of $Ca^{2+}$, but this was irrelevant to the presence of PEG. Therefore, interaction between DNA and PEG during the condensation process (Louie and Serwer 1994) is related to neither electric charge nor superficial molecular-size of transforming DNA.

To examine the effect of PEI, PEG, and $Ca^{2+}$ on attachment of transforming DNA, 2 x $10^7$ protoplasts that passed through the transformation reaction were washed by appropriate buffer, and re-suspended. Nucleic acids were extracted and used as template for PCR that amplify the part of transforming DNA. Presence of PEI (2 kDa, N/P $10^3$) was effective for the protoplasts to retain transforming DNA (Fig. 3), and 200 mM of $Ca^{2+}$ showed a counter effect against PEI. In contrast, the PCR signal was under the limit of detection when protoplasts were incubated in 2 mM of $Ca^{2+}$ without PEG. Addition of 200 mM $Ca^{2+}$ was effective on the attachment of DNA to protoplasts in the absence of PEG, and presence of PEG increased this signal intensity up to 2-fold. These results indicate that deposition of transforming DNA onto protoplasts is enhanced by 200 mM of $Ca^{2+}$, and PEG does not have a major role. In the absence of $Ca^{2+}$, few protoplasts were collected from a reaction mixture containing PEG by centrifugation and subsequent nucleic acid extraction was not possible. Therefore experiments for PEG-treated protoplasts were carried out at 2 mM $Ca^{2+}$ and higher concentrations. At 2 mM of $Ca^{2+}$, transformation frequency was at least one-seventh of that was obtained at 200 mM (Fig. 4**A**).

To understand the role of PEG and $Ca^{2+}$ for transformation, dose-responses to $Ca^{2+}$ were compared between PEG and PEI (Fig. 4A). In the absence of $Ca^{2+}$, both PEI (2 kDa, N/P $10^3$) and PEG (4 kDa, 36.8%) were cytotoxic and viability was less than 0.5%. The protoplast viabilities increased in a dose-responsive manner to $Ca^{2+}$ concentrations, showing a protective effect. Transformation frequencies increased up to 20 mM of $Ca^{2+}$, however, at the higher concentration, an opposite effect was observed between PEI and PEG. Transformation, but not viability, was inhibited in PEI-mediated

transformation at 200 mM of $Ca^{2+}$. This result was consistent with the suppressive effect of 200 mM $Ca^{2+}$ for the DNA-detection assay in the protoplast fraction. Such an effect was not observed in PEG-mediated transformation, rather, the transformation efficiency increased from 20 mM of $Ca^{2+}$. A similar dose-response was also observed with $Mg^{2+}$, but not with $Li^{+}$ (Fig. 4). Apparently in the presence of PEG, viability and transformation showed different responses to both $Ca^{2+}$ and $Mg^{2+}$ compared to PEI.

Responses to different osmotic conditions were dissimilar between PEI and PEG. When the transformation reaction was carried out under 0.5 M sorbitol with PEG, viability decreased by more than 95%, but the effect on the transformation frequency was not as drastic as for the viability (Fig. 5). The transformation frequencies varied between experiments: 80.5 and 17.5 transformants per $10^5$ protoplasts at the first and second experiment, respectively. However the difference in the transformation efficiency was 1.5-fold (9.7 and 6.6 transformants per $10^2$ viable protoplasts). Due to the low viability of protoplasts under 0.5 M sorbitol, we faced difficulty in obtaining a consistent transformation frequency. When averages from the two experiments were compared, about 50-fold higher transformation efficiency was obtained with 0.5 M sorbitol treatment compared to the control 1 M sorbitol. A similar experiment was carried out in triplicate with different mutant strain (DC12; *pyrG89*) and $Ca^{2+}$ concentration (50 mM) and the average transformation efficiency increased 8.3-fold for 0.5 M sorbitol treatment compared to 1 M sorbitol. The effect of a higher osmotic condition (1.5 M sorbitol) with PEG was moderate both on the viability and the transformation frequency.

Similar to the results obtained for the PEG treatment, viability of protoplasts decreased more than 90% under 0.5 M sorbitol with PEI, however the transformation frequency was severely reduced. Therefore a drastic improvement in the transformation efficiency was not observed. The higher osmotic condition did not cause a drastic change for PEI-mediated transformation. Sorbitol was used as osmotic stabilizer during the transformation reaction, and it could be replaced with NaCl and sucrose without affecting transformation efficiency (data not shown).

## Discussion

To increase understanding of the molecular mechanisms of DNA uptake during

transformation of *A. nidulans*, the roles of PEG and $Ca^{2+}$ were examined. By including an autonomously replicating sequence (ARS), AMA1, in the transforming plasmid pTN51, it was possible to avoid chromosomal integration. Presence of this sequence confers a 250-fold difference in transformation frequency (Gems et al. 1991). The major steps involved in transformation with the ARS plasmid include passage through the plasma membrane, cytoplasm, and nuclear envelope, expression of the marker gene, and replication of the DNA. Plasmid encoded *ptrA* and AMA1 are unlikely to influence the process of the transforming DNA in reaching the nucleoplasm once it is taken into a cell, since this is more or less size dependent (Dauty and Verkman 2005). In addition, the experimental conditions used in this study are not expected to affect expression of *ptrA* (Kubodera et al. 2000) and employment of standard growth conditions should keep the efficacy of AMA1 function constant. Therefore, pTN51 is useful for monitoring passage of extracellular DNA into the cell.

Initially, cell and subsequent nuclear fusion was shown to be essential for DNA uptake in *S. cerevisiae* (Harashima et al. 1984). However, it has since been shown that endocytosis, but not cell fusion, is the major pathway for transformation (Burgers and Percival 1987; Neukamm et al. 2002; Kawai et al. 2004). Co-occurrence of DNA uptake and protoplast fusion should result in the appearance of colonies on a plate containing pyrithiamine (the selection marker on pTN51) and lacking both pyrimidine and methionine (selections for the mutations in the two strains used). Although such colonies were obtained, the frequency was less than 3% of the sole-fusion events at 200 mM $Ca^{2+}$ (Table 1). Since the efficiency of fusion was about 2 per $10^2$ viable protoplasts, and that of transformation was 2 and 1 per $10^2$ viable protoplasts of A272 and A773, respectively, the frequency of co-occurrence could be explained by a coincidental event.

Furthermore, since the two mutant strains used in these experiments are derivatives of A4 and the mutations are well-defined (Kafer 1965; May 1989; Hoffmann et al. 2001, and strain information at FGSC), it was inessential to be concerned with the difference in efficiency between fusion of the intra- and inter-mutant strains that could be caused by heterokaryon incompatibility (Saupe and Glass 1997). However, if a segregation of nuclei having different marker mutation occurred at high frequency in the original fused protoplasts, viability of inter-strain fusion would be significantly lower than that of the intra-strain fusion. Since the experiments were not designed to take

intra-strain fusion into account, a concern may arise that the DNA uptake occurs during intra-strain fusion. The difference in appearance of colonies might be an indication of such segregation. However, since the subapical hyphal cells, from which the protoplasts were prepared, contain 3-4 nuclei (Kaminskyj and Hamer 1998), the original fused protoplast shall contain 6-8 nuclei from both strains. If this fused cell starts growing as an apical cell, the probability of all cells, including the apical cells, containing only one type of nuclei would be low. Even if the original fused cell contained 6 nuclei and a septum formed to separate 3 nuclei into two daughter cells, the probability of their containing only one type of nuclei is still low. Therefore as in the case of *S. cerevisiae* (Burgers and Percival 1987), it is concluded that fusion of protoplasts was not required for the uptake of extracellular DNA in *A. nidulans*.

PEI and its derivatives are positively charged hydrophilic polymers and are one of the most efficient non-viral mediators of mammalian transfection (for a review see Neu et al. 2005). Although a controversy may still remain, its role in DNA uptake is well established. Briefly, it electrically interacts with extracellular DNA and subsequent DNA condensation occurs (Dunlap et al. 1997; Kunath et al. 2003). This PEI-DNA complex (polyplex) is incorporated into a cell by endocytosis (Remy-Kristensen et al. 2001). An early endosome is destabilized by proton-sponge effect of PEI and their corruption promotes release of DNA into the cytoplasm as well as preventing degradation in the lysosome (Sonawane et al. 2003; Akinc et al. 2005). In addition, the polyplex is more resistant to attack by DNase (Banerjee et al. 2006).

*A. nidulans* has been transformed by variety of methods from the classic PEG-mediated procedure (Ballance et al. 1983; Yelton et al. 1984) to the use of *Agrobacterium* (Bundock et al. 1995; de Groot et al. 1998), nevertheless there has been no report of utilizing PEI. Transformants were obtained with PEI in *A. nidulans*. The lower viscosity of the transformation mixture, combined with less protoplast aggregation, compared to PEG, can be considered advantageous for analyzing the process of DNA uptake. The effective molecular weight was 2 kDa [similar to that used by Shin et al. (2005)], but the optimal N/P ratio was higher. In human hematopoietic cells, maximum DNA incorporation was observed at N/P 80 and it decreased to less than one-tenth at N/P 300. Although the data varied, similar transformation frequency was obtained between N/P $10^2$ and $10^3$ in *A. nidulans* (Fig. 1). Because N/P ratio of

about 5 is the saturation level [Boussif et al. (1995) and Fig. 2**A**], more excess PEI leads to effective uptake of DNA.

In addition, requirement for $Ca^{2+}$ might be more fundamental. In mammalian transfection system, $Ca^{2+}$ is not included in the reaction. Since instability of endosomes that is caused by PEI relates to both cytotoxicity and efficacy of uptake of DNA, dose-responses to PEI were examined if transformation occurred in the absence of $Ca^{2+}$. Viability of protoplasts decreased by increasing amount of PEI, but no transformant was obtained at N/P $10^2$ at which the viability was adversely affected but some protoplasts were still surviving. At this N/P ratio, transformants were obtained in the presence of 20 mM $Ca^{2+}$. Therefore, although PEI can solely interact with DNA and mediate the attachment to the protoplasts by its own virtue, 20 mM of $Ca^{2+}$ is still required for uptake of DNA in addition to its protective effect against PEI cytotoxicity.

The involvement of $Ca^{2+}$ in the uptake of DNA was also indicated for PEG-mediated transformation, because the dose-responses to $Ca^{2+}$ were different between the viability and transformation frequency. Effect of $Ca^{2+}$ on the protoplast regeneration was saturated at 20 mM, but transformation frequency increased between 20 and 200 mM, indicating this concentration of $Ca^{2+}$ promoted the uptake of DNA. Similar dose-responses were observed between $Ca^{2+}$ and $Mg^{2+}$ for both PEI and PEG, but it was different with $Li^{+}$ (Fig. 4). The transformation efficiency with 50 mM of $Mn^{2+}$ was about one-tenth of that obtained with the same concentration of $Ca^{2+}$ in the presence of PEG (data not shown). Redundancy between $Ca^{2+}$ and $Mg^{2+}$ with an interacting protein has been reported (Mukherjee et al. 2007). Therefore we hypothesize that these divalent cations are physiologically involved in a pathway that mediates the passage of extra-cellular DNA into the cytoplasm as a signal molecule, rather than physically mediate the interaction between protoplasts and DNA with their positive charge.

The occurrence of endocytosis in filamentous fungi had been controversial and still largely remains to be revealed. From this aspect, research on the mode of actions of PEI and PEG during transformation will help elucidate the mechanism of incorporation of macromolecules into fungal cell. The presence of 200 mM $Ca^{2+}$ was inhibitory for PEI-mediated transformation. This was consistent with the result that addition of the same concentration of $Ca^{2+}$ suppressed detection of transforming DNA in the protoplast

fraction (Fig. 3). Therefore a subtle relationship and possible interaction of these positively charged molecules may determine the efficiency of DNA uptake, and this might result in a variation in the transformation frequency (Fig. 1).

When the transformation reaction was carried out with PEG in the presence of 0.5 M sorbitol as an osmotic stabilizer, the transformation efficiency increased about 10- and 50-fold in DC12 and DC25, respectively. In contrast, the effects of other osmotic conditions were moderate. Routine transformation experiments were done at 1 M sorbitol, thus set as a control, and it was not practical to examine a concentration higher than 1.5 M. Therefore it is hard to conclude at this stage that only lower osmotic conditions have such effect. However, considering the buffering function of PEI by its proton-sponge effect and observations that PEG is unlikely to mediate the interaction of transforming DNA with protoplasts by a direct manner (Fig. 2 and 3), this result suggests that PEG is involved in the process inside a protoplast, possibly the stability of endosomes. The requirement of a higher concentration of PEG, two orders of magnitude over to PEI, might support this hypothesis.

Here we report that PEI, which is the basis for non-viral transfection in mammals, also mediates DNA uptake in *A. nidulans*. Not only does it widen the choice for fungal transformation, but also provides knowledge about the mode of action of transformation in filamentous fungi. Since the first report of transformation in *A. nidulans* (Ballance et al. 1983), the protocol utilizing PEG and $Ca^{2+}$ has been practically unchanged for more than twenty years and still widely performed. However, the roles of the key molecules: PEG and $Ca^{2+}$ have remained obscure. Due to the potential of PEG to mediate protoplast fusion (Anne and Peberdy 1975), together with its volume-exclusion effect (Louie and Serwer 1994), its functions have been considered to act outside the cell. In this study, transformation and protoplast fusion were shown to be independent events and PEG was shown not to play a major role in attachment of DNA to the protoplasts. Since PEI promotes transfection by destabilizing endosomes inside a cell as well as by mediating cell-DNA interaction outside the cell, the major role of PEG in fungal transformation may be to promote the release of extracellular DNA in the cytoplasm.

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Figure legends

**Fig. 1** Effect of molecular weight and amount of PEI on transformation of *A. nidulans*. Viability and transformation frequency of protoplasts that were treated with different molecular weights (MW) and amounts of PEI under 20 mM of $Ca^{2+}$ are shown as open and filled boxes, respectively. Amount of PEI included in the transformation reaction is expressed as the N/P ratio (see Materials and methods). The results for 0.8- and 70-kDa PEI are expressed as the mean of 2 consecutive experiments and range to the larger value is shown by a bar. Mean of 5 experiments is shown for 2-kDa PEI with standard deviation by a bar.

**Fig. 2** Interaction of DNA with PEI or PEG and $Ca^{2+}$. **A** Plasmid pBluescript II KS + was incubated in a buffer containing various N/P ratio of PEI, followed by running in 0.8% agarose gel. **B** Effect of $Ca^{2+}$ for interactions between the plasmid pBluescript II KS + and PEG was examined at 0, 2, and 200 mM. Abbreviations: M, molecular size marker (*Hin*dIII digested lambda DNA); D, pBluescript II KS + in TE buffer.

**Fig. 3** Detection of transforming DNA that was co-precipitated with protoplasts. Effect of PEI, PEG and $Ca^{2+}$ on the attachment of DNA to protoplasts during the transformation reaction was examined. A similar transformation reaction but lacking PEG was done as control. Presence of transforming DNA (pTN51) was detected by a 2.2-kb PCR product. Chromosomal DNA (Chr. DNA) was also quantified in all fractions and used as an internal calibration control for the extracted samples.

**Fig. 4** Dose-response of protoplast regeneration and transformation frequency to $Ca^{2+}$, $Mg^{2+}$ and $Li^{+}$ concentration. Viability (open box) and transformation frequency (filled box) of protoplasts that were treated with different concentrations of **A** $Ca^{2+}$, **B** $Mg^{2+}$ and **C** $Li^{+}$ in the presence of PEI (2 kDa, N/P $10^3$) and PEG (4 kDa, 36.8%) are shown. The results are expressed as the mean of 2 consecutive experiments and range to the larger value is shown by a bar.

**Fig. 5** Effect of different osmotic conditions in viability and transformation frequency. Viability (open box) and transformation frequency (filled box) of protoplasts that were

treated with different concentrations of sorbitol in the presence of PEI (2 kDa, N/P $10^3$) and PEG (4 kDa, 36.8%) are shown. The results are expressed as the mean of 2 consecutive experiments and range to the larger value is shown by a bar.

Table 1. Transformation and fusion of two *A. nidulans* mutant strains in the presence of PEG and $Ca^{2+}$

| | $Ca^{2+}$ concentration (mM) | | |
|---|---|---|---|
| | 2 | 20 | 200 |
| Viability[a] | | | |
| A272 | 4.1 ± 0.8 | 9.3 ± 1.8 | 20.8 ± 8.7 |
| A773 | 4.6 ± 3.4 | 13.0 ± 6.8 | 15.5 ± 5.2 |
| Frequency[b] | | | |
| Fusion | 33.5 ± 11.2 | 115.6 ± 92.5 | 178.1 ± 75.8 |
| Fusion and transformation | 0.1 ± 0.1 | 0.8 ± 0.6 | 5.1 ± 2.8 |
| Transformation | | | |
| A272 | 19.1 ± 20.9 | 53.8 ± 40.5 | 187.8 ± 24.9 |
| A773 | 10.5 ± 8.3 | 33.5 ± 20.4 | 79.0 ± 24.3 |

[a] Number of colonies per $10^2$ protoplasts. Mean±standard deviation of four consecutive experiments is shown.

[b] Number of transformants or colonies resulting from protoplast fusion per $10^5$ protoplasts. Mean±standard deviation of four consecutive experiments is shown. Since protoplasts of both mutant strains were in the same reaction, numbers of transformant are shown for each strain and events involving the fusion are shown per sum of both strains.

Fig. 1

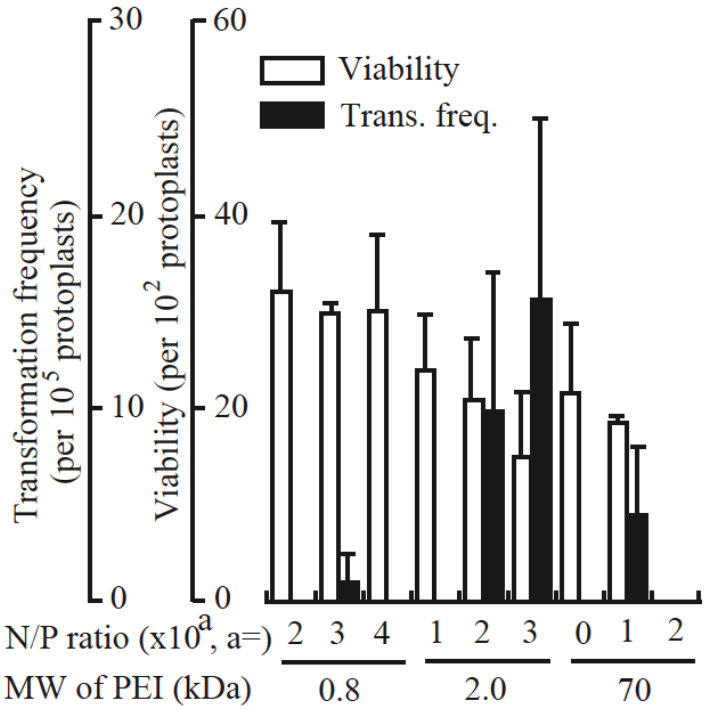

30
20
10
0
60
40
20
0
Viability
Trans. freq.
Transformation frequency (per $10^5$ protoplasts)
Viability (per $10^2$ protoplasts)
N/P ratio (x10$^a$, a=) 2 3 4 1 2 3 0 1 2
MW of PEI (kDa) 0.8 2.0 70

Fig. 2

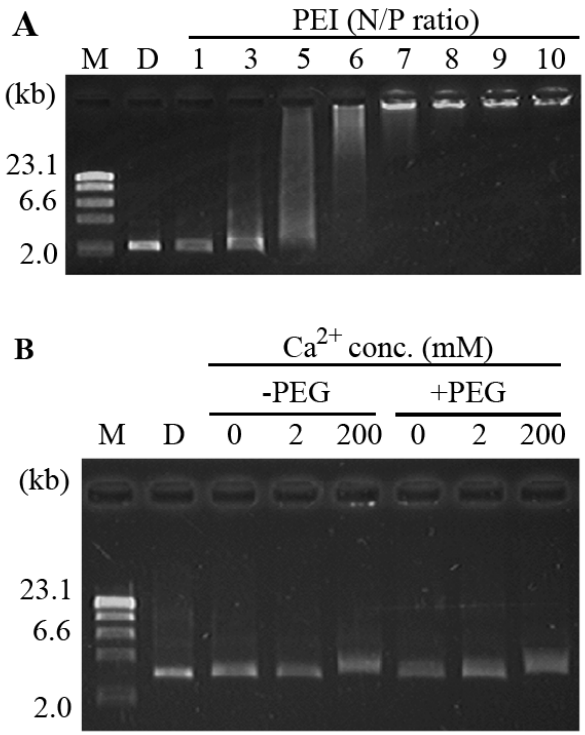

A
PEI (N/P ratio)
M D 1 3 5 6 7 8 9 10
(kb)
23.1
6.6
2.0
B
Ca2+ conc. (mM)
-PEG
+PEG
M D 0 2 200 0 2 200
(kb)
23.1
6.6
2.0

Fig. 3

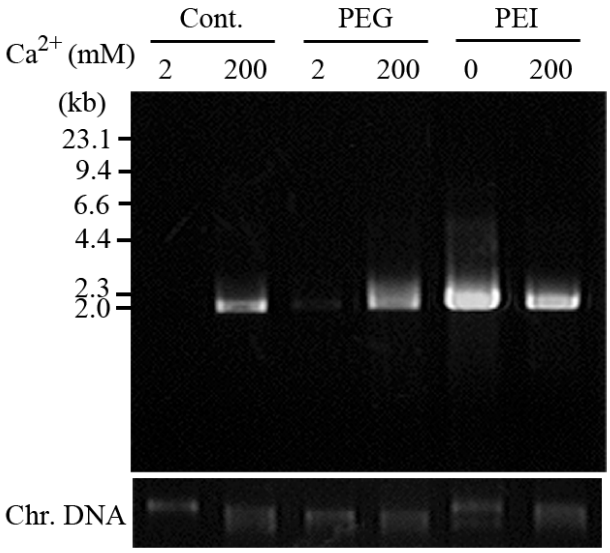


Fig. 4

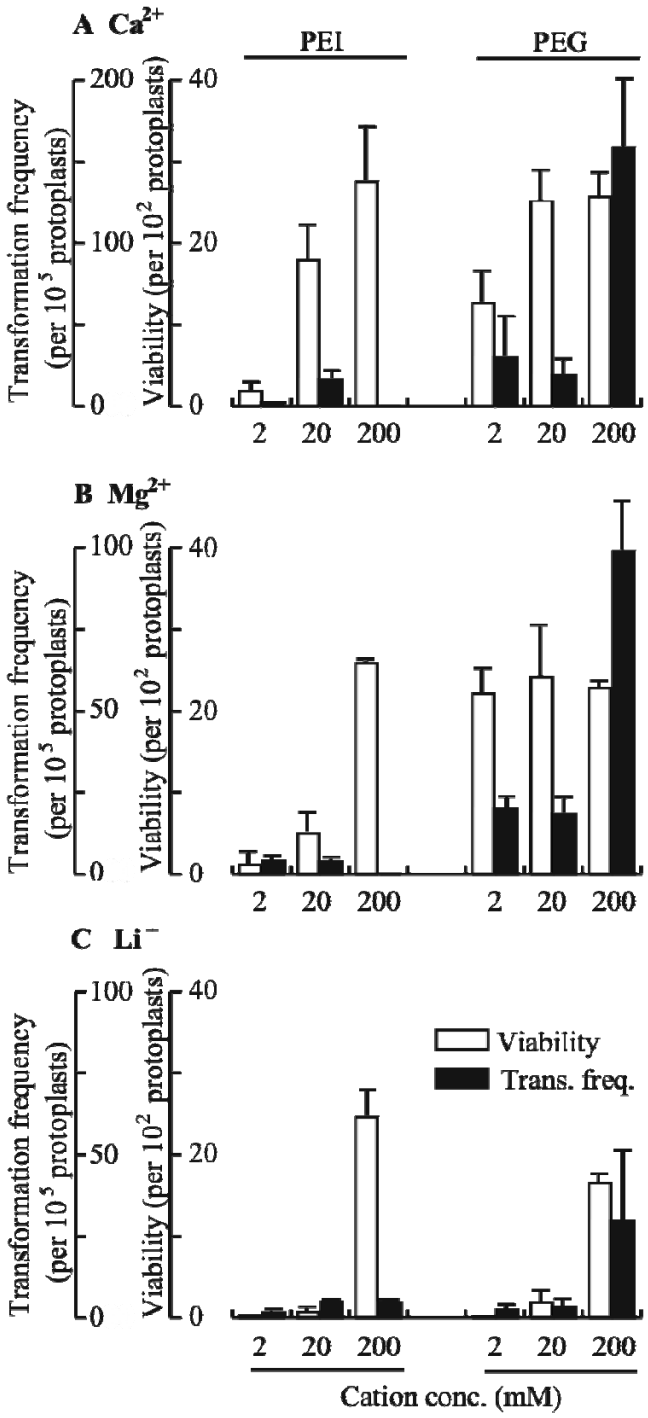
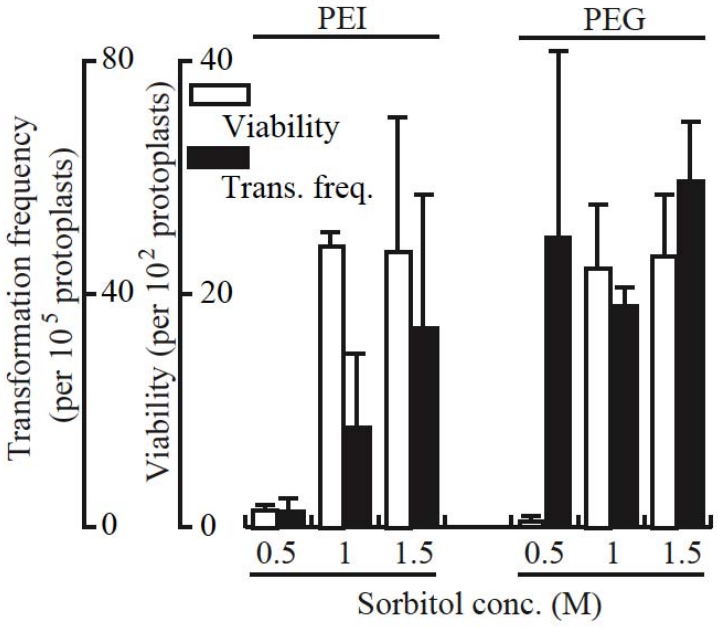

A Ca2+
PEI
PEG
Transformation frequency (per 10^5 protoplasts)
Viability (per 10^2 protoplasts)
200
100
0
40
20
0
2 20 200 2 20 200
B Mg2+
100
50
0
40
20
0
2 20 200 2 20 200
C Li+
100
50
0
40
20
0
Viability
Trans. freq.
2 20 200 2 20 200
Cation conc. (mM)

Fig. 5


PEI
PEG
Transformation frequency (per $10^5$ protoplasts)
Viability (per $10^2$ protoplasts)
80
40
0
40
20
0
Viability
Trans. freq.
0.5
1
1.5
0.5
1
1.5
Sorbitol conc. (M)