Preparation of Protoplasts of *Rhizopus niveus* and Their Transformation with Plasmid DNA

Koji Yanai, Hiroyuki Horiuchi, Masamichi Takagi and Keiji Yano

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received May 7, 1990

An efficient method for protoplast formation of *Rhizopus niveus*, a filamentous fungus with high capability to secrete enzymes out of the cells, was established using three lytic enzymes in combination, and a plasmid with a selection marker for transformation was constructed by transcriptional fusion of a *Rhizopus* promoter with the G418 resistance gene derived from Tn903. Then the protoplasts were mixed with the plasmid DNA in the presence of polyethylene glycol 4000 and CaCl₂. G418-resistant colonies were obtained, indicating that transformation was successful. Southern blot analysis of DNA from a transformant shows that the introduced DNA was present not only integrated into the host chromosome but also replicating extrachromosomally, and that it was rearranged with host DNA during the course of cell cultivation. This is the first report of a transformation system for *Rhizopus*. This system will open the possibility for breeding *R. niveus* at the molecular level.

*Rhizopus*, a filamentous fungus that belongs to zygomycetes, is especially important in the fermentation industry because it secretes a large amount of various enzymes extracellularly, and its culture supernatant from wheat bran is used as a commercial digestive. Therefore, it is very promising to use *R. niveus* as a host to produce valuable proteins using its high ability of protein secretion.

We have isolated genes for secretory proteins such as aspartic proteinase I (RNAP-I) and ribonuclease Rh from the *R. niveus* genome. ¹⁻³ Their signal sequences and promoter regions must be useful for the construction of secretion vectors in *R. niveus* as well as *Saccharomyces cerevisiae*. ⁴ Under the present state of things, construction of an efficient transformation system will contribute to its molecular breeding.

There are many reports concerning the DNA-mediated transformation of filamentous fungi, especially ascomycetes such as *Aspergillus*, ⁵⁻⁷ *Neurospora*, ⁸ *Trichoderma*, ⁹ and *Penicillium*. ¹⁰ On the other hand, in zygomycetes, transformation has been achieved only in *Phycomyces blakesleeanus*, ¹¹,¹² *Mucor circinelloides*, ¹³ and *Absidia glauca*. ¹⁴

This study was undertaken to establish a transformation system for *R. niveus*. We examined the conditions for the formation of *R. niveus* protoplasts and their transformation with plasmid DNA to G418 resistance using the bacterial antibiotic resistance gene under the control of a *Rhizopus* promoter sequence.

Materials and Methods

*Chemicals.* The enzymes used in the preparation of *R. niveus* protoplasts and their sources were: Novozym 234 (Novo Industries A/S, Denmark), chitinase T-1 (Takara Shuzo Co.) and chitosanase (prepared from *Bacillus pumilus*, received as a gift from Meiji Seika Co.). Genticin, referred to throughout the paper as G418, was purchased from Gibco Laboratory.

*Strains and media.* The *R. niveus* strain IFO4810 was used as the recipient in transformation experiments.

Abbreviations:  kb, kilobases; PEG, polyethylene glycol; RNAP, *Rhizopus niveus* aspartic proteinase; SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate (0.15 M NaCl-0.015 M sodium citrate).
Construction, propagation, and amplification of hybrid plasmids were done in Escherichia coli JA221 (recA1 leuB6 trpE6 hisdR* hisdM* lacI lacI thi) and MV1184 (ara D(lac-pro) strA thi (p80 lac Z AM15) D(srl-recA) 306::Tn10 (tet') F'::tra D36 proA B lacZ AM15). E. coli BMH71-18 mutS (D(lac-pro AB) thi supE [F': lacZ AM15 proA B] mutS215::Tn10) was used for site specific mutagenesis. The media for the growth of Rhizopus were as follows. YPG medium was composed of 1% glucose, 2% Polypeptone (Wako Pure Chemical Ind.), and 2% yeast extract (Difco). PD medium was composed of 2.4% potato dextrose broth (Difco). RMM medium was composed of 2% glucose, 0.2% asparagine, 0.05% KH₂PO₄, and 0.025% MgSO₄·7H₂O. E. coli strains were grown in LB medium composed of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl.

Preparation of protoplasts of R. niveus. Sporangiospores were harvested by vigorous shaking of sporangia with mycelia in distilled water from cultures grown in RMM agar medium. Cell debris were removed by filtration through a G-3 filter. Sporangiospores were resuspended at about 5 × 10⁶/ml in YPG medium containing 10 mM proline and germinated at 30°C with shaking for 4-6 hr. Germinated spores (germlings) were filtered through a G-1 glass filter to remove overgrown spores and washed twice with 0.3 M mannitol, 22 mM citric acid, and 55 mM sodium phosphate, pH 5.6. The germlings were added to a lytic enzyme solution dissolved in the same buffer to give a final concentration of about 5 × 10⁶/ml and incubated at 30°C for about 2 hr with shaking.

Transformation of R. niveus. The protoplast suspension was filtered through a G-2 glass filter to remove cell debris, centrifuged at 70 × g for 4 min, washed twice with MMC buffer (0.3 M mannitol, 50 mM CaCl₂, 10 mM MOPS, pH 6.3), and resuspended in the same buffer at a concentration of approximately 10⁷/ml. One hundred µl of protoplasts were added to 10 µl of a solution containing plasmid DNA (25 µg), which had been preincubated with 0.5 mg heparin for at least 20 min on ice, and incubated for 5 min on ice previously. Ten µl of PEG solution (40% (w/v) PEG 4000 in 10 mM MOPS, pH 6.3, and 50 mM CaCl₂) was added and the mixture was incubated for 25 min on ice, gently mixed with 1.25 ml of PEG solution, incubated for 25 min at room temperature, and mixed with 10 ml of MMC buffer. After centrifugation at 70 × g for 4 min, the protoplasts were suspended in 5 ml of YPG medium containing 0.3 M mannitol and incubated for 30 min at 30°C. After centrifugation as described above, the protoplasts were resuspended in 200 µl of the same medium. A sample (100 µl) was added to 5 ml of RMM medium, pH 2.7, containing 0.3 M mannitol and 0.6% agar, which had been held molten at 48°C, then overlayed onto an agar plate of RMM medium, pH 2.7, containing 750 µg/ml of G418. After incubation at 12°C for 15 hr, 5 ml of RMM medium, pH 2.7, containing 0.6% agar and 750 µg/ml of G418 were again overlayed onto the plate, and the plate was incubated at 30°C for 2-3 days.

Southern blot analysis of DNA in a transformant. Total DNA from R. niveus transformants and non-transformed mock control cells was isolated as described previously. A 15-µg sample of the total DNA preparation was digested with a restriction enzyme, fractionated on 0.8% agarose gel, and transferred to a nylon membrane (Hybond-N, Amersham Co.) by the method of Southern. A DNA probe was labelled with 32P-dCTP by the random primer labelling method. Hybridization was done in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 µg/ml of denatured salmon sperm DNA at 68°C for at least 12 hr. The hybridized membrane was washed with 2 × SSC, 0.5% SDS for 5 min, 2 × SSC, 0.1% SDS for 15 min at room temperature, and 0.1 × SSC, 0.5% SDS for 2 hr at 68°C.

Other methods. Preparation of E. coli plasmids, transformation, and analysis by restriction enzymes were done as described previously. In vitro mutagenesis was done by the method of oligonucleotide-directed site specific mutagenesis as described by Morinaga et al.

Results

Formation of protoplasts from R. niveus

To obtain protoplasts from R. niveus enzymatically, a number of factors affecting protoplast formation were examined. First, the effects of mannitol, sorbitol, glucose or MgSO₄ were examined as an osmotic stabilizer for protoplast formation. Protoplasts were efficiently obtained when mannitol, sorbitol, or glucose was used at the concentration of 0.3-0.5 M. In contrast to sugar and sugar alcohols, protoplasts were not obtained when 0.3 M MgSO₄ was used.

Novozym 234, chitinase T-1, and chitosanase were examined singly and in combinations as to their ability to release protoplasts (Table I). No protoplasts were obtained when these lytic enzymes were used alone (A, B and C) and when Novozym 234 and chitinase T-1 were used in combination (D). However, chitosanase released protoplasts from mycelium when used in combination with Novozym 234 or chitinase T-1 (E and F), and the efficiency of protoplast formation was significantly increased when the three enzymes were used all together (G).

The culture age of the mycelium affected the
efficiency of protoplast formation. Protoplasts were obtained most efficiently from the germlings 4-6 hr old. Younger germlings were resistant to lytic enzymes, and the efficiency of protoplast formation from older ones was lower. Photographs of intact germlings 4-6 hr old and of protoplasts prepared from them under the best conditions so far examined are shown in Fig. 1. The protoplasts are perfectly spherical with diameters of 10-20 μm. The regenerating frequency of the protoplasts thus prepared was approximately 4% judging from their colony-forming ability.

Effects of G418 on the growth of R. niveus

A transformation system requires a method by which transformants can be selected from the total regenerating cells. Generally, in filamentous fungi, complementation of a biochemical mutant is used as a selection method. However, in *R. niveus*, there is no combination of a biochemical mutant and a gene complementing it. Therefore, we tried to use drug resistance as a selection marker for *R. niveus* transformation. Effects of various antibiotics such as kanamycin, neomycin, hygromycin B, and cycloheximide on the growth of *R. niveus* were examined, and G418 turned out to inhibit the growth most efficiently. In liquid medium, complete inhibition of growth occurred at the concentration of

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**Table 1. PROTOPLAST FORMATION OF R. niveus USING VARIOUS LYtic ENZYMES SINGLY AND IN COMBINATION**

<table>
<thead>
<tr>
<th>Enzyme solution</th>
<th>Protoplast formation</th>
<th>Incubation time (min)</th>
</tr>
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<tbody>
<tr>
<td>A. Novozym 234 (5 mg/ml)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B. Chitinase T-1 (3 mg/ml)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C. Chitosanase (1.5 mg/ml)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D. Novozym 234 (5 mg/ml) + chitinase T-1 (3 mg/ml)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>E. Novozym 234 (5 mg/ml) + chitosanase (1.5 mg/ml)</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>F. Chitinase T-1 (3 mg/ml) + chitosanase (1.5 mg/ml)</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>G. Novozym 234 (5 mg/ml) + chitinase T-1 (3 mg/ml) + chitosanase (1.5 mg/ml)</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

Germlings were treated at 30°C with each enzyme solution in 0.3 M mannitol buffered at pH 5.6 with 22 mM citric acid and 55 mM sodium phosphate. The conversion rate of germlings into protoplasts was assessed by observation under a microscope. −, ±, +, ++ and +++ indicate 0, <30, 30-60, 60-90, >90% conversion, respectively.

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**Fig. 1. Photographs of Germlings and Protoplasts of R. niveus.**

A) Germling culture at 30°C for 4-6 hr.
B) Protoplasts formed by the three lytic enzymes in combination as shown in Table I. Mannitol (0.3 M) was used as an osmotic stabilizer.
Fig. 2. Effects of G418 on the Growth of *R. niveus* in PD Liquid Medium.

Fifty ml of PD liquid medium containing various concentrations of G418 were inoculated with about $2 \times 10^3$ spores. The cultures were grown at 30°C for 84 hr with shaking, harvested by filtration, and weighed while wet.

![Diagram](image)

**Fig. 3.** Construction of pGGR1.

The 0.8-kb *HindIII–AluI* fragment derived from the 1.7-kb *PvuII* fragment containing the G418 resistance gene was inserted between the *HindIII* and *HincII* site of M13mp9 to give rise to KY-1. KY-2 was obtained by introducing an *EcoRI* site just in front of the initiation codon of G418 resistance gene using site-specific mutagenesis. pGR1 was obtained by ligation of the 0.55-kb *HindIII–EcoRI* fragment of KY-2 and the 2.95-kb *HindIII–EcoRI* fragment of YRpG2.\(^{19}\) pGGR1 was constructed by insertion of the 0.6-kb *EcoRI* fragment, carrying the *R. oryzae* glucoamylase gene promoter, into the *EcoRI* site of pGR1. M13mp9 and YRp7, Tn903-derived DNA and *R. oryzae* DNA are represented by the thin line, open box line, and solid box line, respectively. Restriction enzymes are denoted as follows: A, *AluI*; E, *EcoRI*; H, *HindIII*; P, *PvuII*. 
more than 150 μg/ml of G418 (Fig. 2), but it did not inhibit growth on the agar medium containing as high as 750 μg/ml of G418. This susceptibility of *R. niveus* to G418 in liquid medium indicates that this antibiotic can be used as a selection marker for transformation experiments, since it is possible to test resistance of transformants to G418 by culturing them in liquid medium containing G418.

**Construction of a transforming plasmid, pGGRI**

A plasmid pGGRI for transformation experiments was constructed as shown in Fig. 3. EcoRI site was introduced just before the initiation codon of the Tn903-derived G418 resistance gene by site-directed mutagenesis. To this EcoRI site, the DNA fragment containing *R. oryzae* glucoamylase gene promoter\(^{20}\) was inserted, so that pGGRI contained G418 resistance gene under the control of the *Rhizopus* promoter. It was expected that this chimeric gene would be expressed in *R. niveus*.

**Transformation of *R. niveus* to G418 resistance**

Protoplasts of *R. niveus* were mixed with pGGRI in the presence of PEG4000 and Ca\(^{2+}\) and plated on the selection medium. Even in the case where protoplasts were treated in the same way but without the addition of plasmid DNA, some colonies (mock colonies) appeared. This phenomenon might be due to the incomplete effect of G418 on the growth of *R. niveus* on agar medium, as described previously for the cases of some other fungi.\(^{14}\) However, more colonies appeared upon the addition of pGGRI. To select true transformants out of them, they were transferred one by one onto a plate with fresh selective medium, and the plates were incubated at 30°C to induce sporulation. The spores thus formed were harvested and inoculated into PD liquid medium containing 200 μg/ml of G418. Under these selection conditions, the spores from the mock colonies did not grow, indicating that only the true transformants could be selected by this method.

Southern blot analysis was done to ascertain the presence of the plasmid DNA sequence in one of the transformants selected as described above. \(^{32}\)P-labelled pGR1 (Fig. 3) was used as a probe. No specific hybridization signal was found in EcoRI-digested total DNA of *R. niveus* itself (Fig. 4, lane 8), but a specific hybridization band at 3.5 kb was found in EcoRI-digested total DNA of a transformant (lane 5). However, two bands at 3.6 and 2.9 kb in total DNA digested with HindIII (lane 6) and three bands at 10.5, 4.6 and 3.5 kb in total DNA digested with *SalI* (lane 7), an enzyme which has no cutting site in pGGRI, were found. These results suggested that the transformant had acquired the plasmid DNA sequence and that the recombination of the introduced DNA with the genomic DNA had occurred, since the restriction enzyme digestion patterns were different between the plasmid DNA and the total DNA of the transformant. In the DNA sample without enzyme digestion (lane 4), five bands were found. Although the
position of the uppermost band corresponded to the chromosomal DNA region, the other four lower additional bands clearly represented plasmid DNA. This result suggested that the introduced DNA was not only integrated into the chromosomal DNA, but also present as plasmid DNA autonomously replicated extrachromosomally. However, attempts to transform *E. coli* to ampicillin resistance by introducing total DNA isolated from this transformant were unsuccessful. This may be due to the disruption of the ampicillin resistance gene or the origin of replication in *E. coli* contained in pGGR1 by recombination in *R. niveus*.

The transformant described above was further cultivated for 96 hr, and the total DNA was prepared from the cells at an interval of 24 hr. Southern blot analysis of these DNA samples indicated that rearrangement of DNA occurred rather frequently.

**Discussion**

The main objective of this study was to establish a transformation system for *R. niveus*. One of the key steps for this was protoplast formation, since in filamentous fungi, the lithium acetate method, which doesn’t require protoplasts, cannot be used except for a few examples.21 - 23)

Most fungi contain glucan and mannan as the main cell wall components. On the other hand, the cell wall of zygomycetes contains chitosan as one of the main components.24)

There are several reports concerning the protoplast formation of zygomycetes, and chitosanase activity was required in each case.25 - 29)

In this study, an efficient method for protoplast formation of *R. niveus* was established using three lytic enzymes, that is, Novozym 234, chitinase T-1, and chitosanase in combination. No protoplasts were released when chitosanase alone was used, but a low frequency protoplast formation was observed when it was used in combination with Novozym 234 or chitinase T-1. These results indicated that chitosanase activity was required but not sufficient for protoplast formation of *R. niveus* as well as other zygomycetes. Both Novozym 234 and chitinase T-1 are also required for efficient protoplast formation. By using chitinase T-1, degradation of chitin, which is another main cell wall component of zygomycetes, may increase the efficiency of protoplast formation. Novozym 234 contains various enzymatic activities and those of chitinase, glucanase, and protease are especially strong.30) At present, we don’t know what activities in Novozym 234 are responsible for the efficient protoplast formation. There is a report describing the role of protease activity in the degradation of *Rhizopus* cell walls.31) The protease activity in Novozym 234 may be involved in the efficient protoplast formation.

Transformation of the protoplasts thus prepared was successful by mixing the protoplasts in the presence of PEG 4000 and Ca^{2+} with DNA of the plasmid pGGR1. As the effect of G418 on the growth of *R. niveus* on an agar plate was not complete, culturing of the spores in liquid medium containing G418 was required to select true G418-resistant transformants. This procedure is time-consuming and not suitable for examining various conditions affecting the transformation frequency or for cloning genes by complementation experiments. In an improved system, transformants should be selected by plating the cells on a agar plate only once. Therefore, construction of a transformation system is expected in the future using a gene to make the cells resistant to an antibiotic that inhibits the growth of *R. niveus* on an agar plate completely, or a gene that complements a biochemical mutant available in *R. niveus*.

Southern blot analysis of the total DNA from one of the transformants suggested that the introduced DNA was rearranged and present not only integrated into the host chromosome but also replicating extrachromosomally. Two explanations for the presence of the introduced DNA in the two different states are possible: one is that the introduced DNA being replicated extrachromosomally may be...
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partly integrated into the host chromosome, and the other is that all the introduced DNA first being integrated into the host chromosome may be partly excised from it by recombination during the course of cell growth.

The states of the introduced DNA in the transformant cells obtained in this study are unique, since the stable integration of introduced DNA into the host chromosome is general in filamentous fungi. However, although there are only limited reports on transformation systems of zygomycetes, the introduced DNA has been found to be replicated autonomously in this group of organisms. In these cases, autonomous replication is thought to be driven either by an ARS element selected in *Saccharomyces cerevisiae* or by the element associated by chance with the structural gene contained in the plasmid.

Recently, integrative transformation of *A. glauca* was reported, but in this case repetitive DNA had to be inserted into a vector DNA. These results taken together may suggest that the frequency of integration of a vector DNA into the chromosome is lower in zygomycetes than in other filamentous fungi. This may be true with *R. niveus*.

In this paper, we have shown that the introduction of DNA into *R. niveus* is possible. This system will enable us to study the heterologous gene expression and protein secretion in *R. niveus*.

Acknowledgments. The authors are indebted to Dr. Sakaguchi, Nihon Shokuhin Kako Co., Ltd. for his kind support and discussion. Plasmid DNA of YRpG2 was kindly provided by Prof. Takahashi, Institute of Applied Microbiology, the University of Tokyo. The promoter DNA of the glucoamylase gene of *R. oryzae* was kindly given by Suntory Co., Ltd., chitosanase was kindly given by Meiji Seika Co., Ltd. and chitinase T-1 was kindly given Asahi Kogyo Co., Ltd.

References


