An efficient genetic manipulation protocol for dark septate endophyte *Falciphora oryzae*

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Abstract

**Objective** To investigate the protoplast preparation and transformation system of endophytic fungus *Falciphora oryzae*.

**Results** *F. oryzae* strain obtained higher protoplast yield and effective transformation when treated with enzyme digestion solution containing 0.9 M KCl solution and 10 mg mL\(^{-1}\) glucanase at 30 °C with shaking at 80 rpm for 2–3 h. When the protoplasts were plated on a regenerations-agar medium containing 1 M sucrose, the re-growth rate of protoplasts was the highest. We successfully acquired green fluorescent protein-expressing transformants by transforming the pKD6-GFP vector into protoplasts. Further, the GFP expression in fungal hyphae possessed good stability and intensity during symbiosis in rice roots.

**Conclusions** This study provided a protoplast transformation system of *F. oryzae*, creating opportunities for future genetic research in other endophytic fungi.

**Keywords** Endophytic fungus · *Falciphora oryzae* · Protoplast transformation · Symbiosis

Introduction

Beneficial relationships between plants and endophytes commonly occur in ecosystems. Endophytic fungi play essential roles in improving plant growth and performance under biotic and abiotic stress. *Faciphora oryzae* was first described as a beneficial dark septate endophyte residing in domestic Chinese wild rice (*Oryza granulata*) (Yuan et al. 2010). *F. oryzae* could vigorously promote rice growth and induce systemic resistance to rice blast (Su et al. 2013). Comparative genomic and transcriptomic analyses further showed that *F. oryzae* evolved from a pathogenic ancestor by the gain or loss of orphan genes, DNA duplications, gene family expansions, and the frequent translocation of transposon-like elements (Xu et al. 2014). The biological roles and evolutionary genetics render *F. oryzae* an attractive model for studying the endophytic interaction with plants. To date, few endophytic fungi have...
stable genetic transformation systems. Therefore, we intend to explore a genetic transformation method for *F. oryzae*.

Versatile genetic methods have been developed and applied on filamentous fungi, such as *Agrobacterium tumefaciens*-mediated transformation, polyethylene glycol-mediated transformation, electroporation, and particle bombardment. The protoplast-mediated transformation is a commonly used fungal transformation method. The principle is to use commercially available enzymes to remove fungal cell walls for generating protoplasts. Subsequently, some chemical reagents (such as PEG) promote the fusion of exogenous nucleic acids and protoplasts. Since the fungal cell wall components are highly variable among different strains, no universal transformation method can be applied to different fungal strains.

Moreover, due to the limited understanding of cell wall hydrolases, the protoplast preparation can hardly be standardized. Therefore, the development of an optimized protoplast-mediated transformation method for fungi still requires significant effort. In this study, the key factors affecting the protoplast yield were optimized. We explored the PEG-mediated protoplast transformation as a tool for the genetic transformation of *F. oryzae*. And we successfully got GFP-expressing transformants of *F. oryzae* through this highly efficient transformation method.

**Materials and methods**

Strains culture and vectors

*F. oryzae* strain (CCTCC M 2021505) was cultivated on potato dextrose agar (PDA) medium for 7 days in the dark. Then four fungal plugs (5 mm each) were picked out and inoculated into a conical glass flask containing 150 mL of potato dextrose broth (PDB) (with 5 g glucose/L) (Sivasithamparam 1975). The flasks were kept on a shaker (150 rpm) at 25 °C for 3 days (Liu et al. 2016).

The plasmid used for transformation was pKD6-GFP (Li et al. 2012). pKD6-GFP contains a sulfonylurea gene with superoxide dismutase 1 promoter and trpC terminator from *Magnaporthe oryzae*. Then the pKD6-GFP vector was digested by *SspI* to be linearized DNA for protoplast transformation.

Antibiotic resistance screening of *F. oryzae*

Fungal plugs were inoculated on a PDA medium supplemented with different concentrations of SUR (from 0 to 300 μg mL⁻¹) to determine the sensitivity of *F. oryzae* towards SUR. Petri dishes were incubated for 15 days at 25 °C. The minimum resistance of SUR was evaluated.

Protoplast isolation, purification, and regeneration of protoplasts

*F. oryzae* strain was cultured on PDA plates at 25 °C for 10 days. Asexual spores (conidia) from one plate were harvested by gently scraping the agar with sterile distilled water. The resulting spore suspension was filtered through a sterile Miracloth filter (EMD Millipore, USA). The filtered spore suspension was inoculated in 50 mL of complete medium (CM) and incubated at 25 °C and 180 rpm for 72 h. Mycelia were collected by filtering through a Miracloth filter and then washed three times with 0.9 M KCl. In parallel, 0.1 g of glucanase (Bide Biotech Company, China) was dissolved in 10 mL of 0.9 M KCl solution and filtered through a 0.22 μm filter (Millex®GP, EMD Millipore, USA). This filtered solution was added to the Erlenmeyer flask containing resuspended mycelia, and the mixture was incubated at 30 °C, 80 rpm. Protoplast release was checked every 30 min by counting in the Neubauer chamber. Released protoplasts were carefully filtered two times, using sterile Miracloth and a sterile 40-μm nylon filter. The initial protoplasts were washed by cold 0.9 M KCl solution twice and cold STC solution [1 M sucrose, 0.5 M Tris-HCl (pH 7.0) and 0.5 M CaCl₂], and centrifuged at 4 °C (3000 rpm, 10 min). Finally, the protoplasts were resuspended gently in 10–20 mL STC solution, and the concentration was adjusted to 10⁸ mL⁻¹. For regeneration, the protoplasts were diluted with STC solution and grown on a regenerations-agar (RgA) medium (PDA medium with 1 M sucrose) at 25 °C for 4 days. The protoplast diluted with sterile water was used as a control. The number of colonies growing on the culture medium was counted after 4 days. Three kinds of cell wall degrading enzymes such as lysing enzymes from *Trichoderma harzianum* L1412 (Sigma, USA), snailase (Sigma, USA), and glucanase (Bide Biotech Company, China) were used.
Protoplast transformation and microscope

Protoplast transformation of *F. oryzae* was carried out by PEG/CaCl₂ method. First, the fungal protoplasts of 150 μL were placed on ice for 10 min. Then, the protoplasts were added with 1 μL of heparin solution (10 mg mL⁻¹) and 3–5 ug linearized plasmid, iced for 10 min. Next, the STC-PEG (SPTG: 4 g PEG4000 dissolved in 10 mL STC solution) was mixed gently and placed on the ice for 15 min. Finally, the transformed protoplasts were cultured on the regenerated medium containing sulfonylurea for 5–7 days, and then transferred to the selective medium with sulfonylurea for another 3–5 days. The overexpression transformants were observed under a Nikon fluorescence microscope.

Co-cultivation of *F. oryzae* and rice

The stability and intensity of GFP expression in *F. oryzae* were assayed by co-culturing *F. oryzae* and rice in tissue culture bottles. Rice seeds were surface-sterilized in 1% sodium hypochlorite solution for 10 min, then rinsed in sterile water. They were then planted in solid Murashige & Skoog medium and inoculated with *F. oryzae* strain plugs (eight seeds, four fungal plugs per bottle). The plants were kept with a 16 h light/8 h dark photoperiod at 24/22 °C for 20 days. The green fluorescence was detected at 515 nm using an excitation wavelength of 488 nm under an LSM780 laser scanning confocal microscope.

Statistical analysis

Each experiment was performed in triplicates. The data were statistically analyzed using SPSS version 16.0 software (SPSS Inc., USA) and are presented as the mean ± standard deviation (SD). Graphs were created using GraphPad Prism 8.

**Results**

Screening of optimum conditions for isolation and regeneration of protoplasts

Digestion enzyme is a crucial factor controlling the efficiency of protoplast transformation. Three cell wall digesting enzymes (glucanase, lysing enzyme, and snailase) were tested alone or combined to release protoplasts from *F. oryzae*. The results showed that glucanase had a higher yield of protoplasts than the other two enzymes (Fig. 1A). Furthermore, the combination of enzymes was more efficient than a single enzyme. The combination of these three enzymes yielded the highest protoplasts, followed by the combination of glucanase and snailase (Fig. 1A). Considering the operability and cost, the use of glucanase alone also can produce sufficient protoplasts for transformation.

The number of fungal protoplasts released gradually increased along with glucanase concentration and reached a maximum of 10 mg mL⁻¹ (Fig. 1B). The volume of enzyme solution used for digestion also had a great influence on protoplast production. It was found that the protoplast yield increased along with the volume of enzyme digestion solution, reaching the highest yield at 40 mL of glucanase at 10 mg mL⁻¹ (Fig. 1C).

The number of protoplasts released gradually increased with the increase in incubation time. After 3 h incubation, the release of protoplasts reached a peak at 12.6 × 10⁷ g⁻¹ (Fig. 1D). Prolonging the incubation time did not increase the yield of protoplasts significantly. However, it caused a damage to the plasma membrane and affected the quality of protoplasts (Liu et al. 2010). The yield of protoplasts released from *F. oryzae* showed no apparent differences when the digestion reactions were incubated at a temperature between 30 °C and 32 °C. Fewer protoplasts were produced when incubating at temperatures lower than 30 °C and higher than 32 °C (Fig. 1E).

The fungal age also affected the release of protoplasts. The cell walls of mycelium are thickened with age, making it challenging to release protoplasts. However, the hyphae in the logarithmic growth stage have stable metabolic activity and strong adaptability. In this work, 4-day-old hyphae of *F. oryzae* were broken into fragments and re-cultured in a liquid complete medium (CM) medium to ensure that the hyphae were young during logarithmic growth phase. The yield of the protoplasts increased continuously from day 1 to 3, and it was stable on day 4. The second day is the best appropriate with a high yield (Fig. 1F).

Additionally, the osmotic pressure stabilizers play crucial roles in protoplast isolation and regeneration (Liu et al. 2010). Four different osmotic pressure
stabilizers (NaCl, KCl, sucrose, and sorbitol) were tested. The results showed that protoplast yield reached the highest (12.47 ± 0.75) when 0.9 M KCl was used as an osmotic pressure stabilizer (Table 1).
Furthermore, the osmotic pressure stabilizer in the regeneration medium plays a fundamental role in the re-growth of protoplasts. The results also showed that the protoplast regeneration rate reached highest \( (42.2 \pm 2.67) \) when RgA medium containing 1 M sucrose.

Table 1: Effect of osmotic pressure stabilizers for protoplast formation and regeneration

| Osmotic stabilizers | Digestion solution | RgA medium |
|--------------------|--------------------|------------|
|                    | Concentration (mol L\(^{-1}\)) | Protoplast yield \( (10^7 \text{ g}^{-1}) \) | Regeneration cells \( (10^6 \text{ g}^{-1}) \) | Protoplast regeneration rate (%) | Protoplast regeneration rate (%) |
| KCl                | 0.8                | 10.77 ± 0.55\(^{b}\) | 6.37 ± 0.42\(^{c}\) | 5.91 ± 0.33 | 0.8 | 19.03 ± 0.35\(^f\) |
|                    | 0.9                | 12.47 ± 0.75\(^a\) | 21.80 ± 1.32\(^a\) | 17.48 ± 0.66 | 1  | 26.93 ± 1.46\(^d\) |
| NaCl               | 0.8                | 1.6 ± 0.26\(^{gh}\) | 0.37 ± 0.06\(^{f}\) | 2.29 ± 0.16 | 0.8 | 6.43 ± 0.75\(^i\) |
|                    | 0.9                | 2.13 ± 0.40\(^{f}\) | 0.83 ± 0.06\(^{ef}\) | 3.9 ± 0.62 | 1  | 6.73 ± 1.00\(^{d}\) |
|                    | 1                  | 1.67 ± 0.32\(^{fg}\) | 0.87 ± 0.06\(^{ef}\) | 5.2 ± 0.34 | 1.2 | 3.53 ± 0.67\(^{j}\) |
| Sucrose            | 0.6                | 3.67 ± 0.32\(^{e}\) | 5.80 ± 0.2\(^{c}\) | 15.81 ± 0.81 | 1.2 | 37.77 ± 1.29\(^{b}\) |
| Sorbitol           | 0.4                | 0.83 ± 0.06\(^{h}\) | 0.57 ± 0.06\(^{ef}\) | 6.8 ± 0.45 | 0.8 | 15.77 ± 1.56\(^{gh}\) |
|                    | 0.5                | 1.13 ± 0.15\(^{gh}\) | 1.23 ± 0.15\(^{e}\) | 10.88 ± 0.23 | 1  | 21.93 ± 1.46\(^{e}\) |
|                    | 0.6                | 0.93 ± 0.12\(^{gh}\) | 0.53 ± 0.06\(^{ef}\) | 5.71 ± 0.42 | 1.2 | 13.3 ± 1.95\(^{h}\) |

The bold fonts indicate the best concentration of osmotic pressure stabilizers for protoplasts formation and regeneration, which showed significant differences from the others. Significant differences (One-way ANOVA): lowercase letters, \( P < 0.05 \). The experiment was repeated three times with similar results.

Antibiotic resistance and transformation of GFP-vector in \( F. \text{ oryzae} \)

Sulfonylurea resistance genes were tested as a selection marker. The minimal inhibitory concentration was determined as the lowest concentration at which no visible hyphal growth was observed. Our analysis showed that the colony growth was completely inhibited when the sulfonylurea concentration reached 300 \( \mu \text{g mL}^{-1} \) (Fig. 2A, B), suggesting that the minimum inhibitory concentration (MIC) value for \( F. \text{ oryzae} \) was 300 \( \mu \text{g mL}^{-1} \). We thus used this antibiotic concentration for the selection of \( F. \text{ oryzae} \) transformants generated from the protoplast transformation.

Fluorescence expression vector pKD6-GFP was transfected into the protoplasts of \( F. \text{ oryzae} \). First, the GFP-tagged transformants were grown on a dehydrated culture (DCM) medium supplemented with 1 M sucrose and 300 \( \mu \text{g mL}^{-1} \) sulfonylurea for 5–7 days. Then, the regenerated transformants were re-cultured on a DCM containing 300 \( \mu \text{g mL}^{-1} \) of sulfonylurea for two generations. Fluorescence observation showed that GFP was strongly expressed in hyphae and conidia from three generations of transformants (Fig. 3). The results suggested that...
exogenous promoter superoxide dismutase 1 also has a strong ability to start the gene expression in *F. oryzae*.

The colonization of GFP-expressing *F. oryzae* in rice roots

To further clarify the intensity and stability of fluorescence expression, we inoculated the GFP-labeled *F. oryzae* strain on rice roots. It was found that *F. oryzae* successfully infected the rice roots, gradually spread from the epidermis to the cortex, and finally reached the endodermis (Fig. 4). Moreover, in the co-culture process of rice and *F. oryzae*, the fluorescence expression in mycelium was stable and coherent. Furthermore, the intensity of fluorescence expression was vigorous, facilitating the mycelial observation in root tissue (Fig. 4).

**Discussion**

Endophytic fungi have attracted great interest because of their excellent gene pool, which can be used in agricultural and industrial fields. Genetic transformation techniques are a premise to target and modify genes efficiently and reveal the function of target genes. The method to deliver foreign nucleic acid into cells is the sticking point for fungal genome modification.

Here, the PEG-mediated protoplast transformation of *F. oryzae* was reported for the first time and had a high transformation efficiency. To better understand the functional genomics and molecular mechanism in *F. oryzae*, we transformed the vector of pKD6-GFP into *F. oryzae* by this approach. The GFP fluorescence transformants exhibited excellent efficiency in the protoplast system.

![Fig. 3 The fluorescence in hyphae and conidia from three generations of transformants pKD6-GFP. Scale bar = 50 μm](image)
An effective transformation system is a prerequisite for studying fungal genetic manipulation and functional genomics (Dobrowolska and Staczek 2009). Agrobacterium tumefaciens-mediated transformation is widely used for transforming various fungal materials (Groot et al. 1998). In *F. oryzae*, Agrobacterium tumefaciens-mediated transformation has been used for targeted gene deletion (Liu et al. 2016), but it is laborious and multi-step. PEG-mediated protoplast transformation has been an ideal method for fungal genetic transformation because of its high efficiency and simplicity (Liu and Friesen 2012). Protoplast transformation was first applied in *Saccharomyces cerevisiae* using snailase for degrading cell walls and sorbitol for preserving protoplasts (Hutchison and Hartwell 1967). Later, this method was commonly used in filamentous fungi, such as *Neurospora crassa* (Case et al. 1979), *Aspergillus nidulans* (Tilburn et al. 1983), and *Ustilago esculenta* (Yu et al. 2015). Although these transformation methods have been improved, the basic steps remain essentially the same. However, due to many fungal species and their complex cell wall component, there is no universal transformation method suitable for different fungal strains. Species-specific transformation protocols must be optimized for each strain. Thus, we conducted a comprehensive analysis of the protoplast transformation system of *F. oryzae*, and estimated that 100 mg of fresh fungal hyphae digested with 10 mg mL\(^{-1}\) of glucanase in 40 mL solution containing 0.9 M KCl for 4 h could generate approximately \(4 \times 10^8\) protoplasts. Protoplasts regenerated on RgA medium containing 1 M sucrose.

Several factors are crucial for the success of protoplast transformation. Firstly, enzymic digestion of the fungal cell walls is pivotal to produce protoplasts. The components of the fungal cell walls are highly complex and dynamic and vary among fungi species. Therefore, selecting an appropriate cell wall degrading enzyme and its concentration is vital for protoplast acquisition. Secondly, fungal material and age are also crucial factors. Protoplasts can also be prepared from hyphae (Vollmer and Yanofsky 1986) and conidia (Yu et al. 2015). The fungal hyphae in the logarithmic phase appear to be more sensitive to the suitable digestion enzymes and are more easily degraded to remove the cell wall. In this study, the 4-h-old newly born hyphae of *F. oryzae* are enzymatically hydrolyzed for protoplast preparation. Thirdly, it is also vital to choose a suitable osmotic stabilizer for protoplasts to maintain a stable osmotic pressure, avoiding protoplast deformation or rupture. Commonly used osmotic stabilizers are sucrose, sorbitol, sodium chloride, and potassium chloride, etc. Sorbitol solution with a concentration of 0.8–1.2 M was used for the protoplast preparation of *N. crassa* (Case et al. 1979), *Aspergillus sp.* (Tilburn et al. 1983), and *Trichoderma sp.* (Dobrowolska and Staczek 2009) to maintain the osmotic stability of protoplasts.

The development of genetic transformation techniques is a breakthrough in our attempt to modify fungal genes. This technique enables scientists to target and modify genes efficiently to reveal the functions of targeted genes or insert new genetic elements into the genomes of the strains, such as promoters, to modify the expression of endogenous genes. The selection of appropriate promoters is also an essential factor for the success of the genetic transformation. Here, the superoxide dismutase 1 gene is highly expressed at various stages of fungal development. Enhanced green fluorescent protein (eGFP) with superoxide dismutase 1 promoter was expressed uniformly, firmly, and stably in hyphae and

**Fig. 4** Colonization of *F. oryzae* in rice roots. The rice roots were co-cultivated with *F. oryzae* for 20 days. In a root cross-section, GFP-tagged hyphae gradually extended from the epidermis to the cortex without penetrating the stele. The green fluorescence was from GFP-tagged hyphae, and the red fluorescence was from spontaneous fluorescence of roots. Scale bar = 100 μm.
conidia of *F. oryzae*. During the symbiosis process with rice, the fluorescence expression was stable and coherent in the mycelium. Furthermore, the intensity of fluorescence expression was strong enough that the spontaneous fluorescence did not obscure it from plant roots. Thus, these two advantages make superoxide dismutase 1 to be an excellent promoter choice.

This method may be used to investigate several functional genes for future molecular pathways and symbiotic processes. The effective genetic transformation system also creates opportunities for future genetic research in other endophytic fungi.

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**Author contribution** Conceptualization and Methodology: Lin FC and Su ZZ; Data curation: Su ZZ and Dai MD; Writing-Original draft preparation: Su ZZ and Dai MD; Investigation: Su ZZ, Zhu JN, Zeng YL, Lu XJ, Liu XH; Funding acquisition: Lin FC and Su ZZ; Resources: Lin FC and Su ZZ; Supervision: Lin FC; Writing- Reviewing and Editing: Lin FC and Su ZZ.

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**Declarations**

**Conflict of interest** All authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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