An Efficient Genetic Manipulation Protocol for Dark Septate Endophyte *Falciphora Oryzae*

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Research Article

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Abstract

Falciphora oryzae is a dark septate endophyte (DSE) isolated from wild rice roots (*Oryza sativa* L.). It was classified as a non-clavicitaceous endophyte. The fungus colonizes rice roots, showing a significant increase in agronomic parameters with plant biomass, rice blast resistance, yield, and quality. The construction of the genetic manipulation system is critical to study the relationship between *F. oryzae* and *O. sativa*. In the present study, the protoplast preparation and transformation system of *F. oryzae* was investigated. The key parameters affecting the efficiency of protoplast production, such as osmotic pressure stabilizer, enzyme digestion conditions, and fungal age, were studied. The results showed that *F. oryzae* strain obtained higher protoplast yield and effective transformation when treated with enzyme digestion solution containing 0.9mol L^{-1} KCl solution and 10 mg mL^{-1} glucanase at 30℃ with shaking 80 rpm for 2-3 h. When the protoplasts were plated on a regenerations-agar (RgA) medium containing 1M sucrose, the re-growth rate of protoplasts was the highest. We successfully acquired GFP-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.The genetic manipulation system of endophytic fungus facilitates the further exploration the interaction between the endophytic fungus and their hosts.

Introduction

Beneficial relationships between plants and endophytes commonly occur in the ecosystems and play essential roles in improving plant growth and helping plants overcome biotic or abiotic stress. *Faciphora oryzae* was first described as a beneficial dark septate endophyte (DSE) 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). Phylogenetic analyses have shown that *F. oryzae* has a close relationship to other members in *Magnaporthaceae*, such as *Gaeumannomyces* and *Magnaporthe*, most of which are plant pathogens. 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 role and evolutionary genetics render *F. oryzae* to be an attractive model for exploring the mutualistic interaction of endophytes and plants. To date, few endophytic fungi have been sequenced with a whole genome, let alone have stable genetic transformation systems. Therefore, in this study, we intend to explore a genetic transformation method of *F. oryzae* to study the interaction between other endophytic fungus and host plants.

In recent years, versatile genetic methods have been developed and applied on filamentous fungi for industrial and agricultural application, such as *Agrobacterium tumefaciens*-mediated transformation (ATMT), PEG-mediated transformation, electroporation, and particle bombardment (Mullins et al. 2001; Amey et al. 2002). Protoplast-mediated transformation systems are widely used in various fungi (Brachmann et al. 2004; Brown et al.1998; Betts et al. 2007). The common methods of protoplast genetic transformation include electro-transformation and polyethylene glycol (PEG)-mediated transformation (Bates 1994). Electro-transformation is a simple and effective method to make foreign genes enter the cell membrane through a high voltage ratio, and the electroporator is essential (Faber et al. 1994). PEG-mediated transformation is to change the permeability of cell membrane by PEG treatment and promote the entry of foreign DNA into the protoplast. The method requires no special equipment, and the result is relatively stable (Liu et al. 2010; Terfrüchte et al. 2014).

PMT is the most commonly used fungal transformation method, which relies on many competent fungal protoplasts. The principle is to use some commercially available enzymes to remove fungal complex cell wall components for generating protoplasts. Subsequently, some chemical reagents (such as PEG) are used to promote the fusion of exogenous nucleic acids and protoplasts, as described in more detail below. The components of the fungal cell wall are highly variable among different strains. Even components of the spore coat are significantly different from that of hyphae from the same strain. Thus, there is no universal transformation method that can be applied to different fungal strains. Due to the limited understanding of cell wall hydrolases, the protoplast preparation can hardly be standardized. Therefore, the development of an optimized PMT method for fungi still requires significant effort. In this study, the key factors affecting the protoplast yield were optimized. Then, we explored the PEG-mediated protoplast transformation as a tool for the genetic transformation of *F. oryzae*. The highly efficient transformation method we developed enables us to obtain a large number of transformants. And we successfully got GFP-expressing mutants 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 PDA for 7 days in the dark. Then four fungal plugs (5 mm each) were picked out and inoculated into a glass flask containing 150 ml of potato dextrose broth (PDB, with 5g 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 (SUR) gene as a dominant selectable marker under the control of SOD1 promoter and trpC terminator from *M. oryzae*. And pKD6-GFP vector was digested by SspI to be linearized DNA for protoplast transformation (Oh and Chater 1997). *Escherichia coli* DH5α was used for propagating all DNA manipulations.

Antibiotic resistance screening of *F. oryzae*

Fungal plugs were inoculated on PDA medium supplemented with different concentrations of SUR (from 0 to 300 μg ml^{-1}) to determine the sensitivity of *F. oryzae* toward SUR. 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 (CCTCC M 2021505) was cultured on PDA plates at 25°C for 10 days. Asexual spores (conidia) of 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 CM medium and incubated at 25°C and 180 rpm for 72 h. Mycelia obtained was collected by filtration in Miracloth filter and washed three times with 0.9 M KCl. In parallel, 0.1 g of glucanase (Bide Biotech Company, China) were dissolved in 10 ml of 0.9 M KCl solution and filtered through 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, orderly using sterile Miracloth and a sterile 40-μm nylon filter. The initial protoplast was washed by cold 0.9 M KCl solution twice and cold STC solution again [1 M sucrose, 0.5 M Tris-HCl (pH 7.0) and 0.5 M $CaCl_2$)], and centrifuged at 4 ˚C (3000 rpm, 10min). Finally, the protoplasts were resuspended gently in 10-20 mL STC solution and adjusted the concentration at 10^8 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 the control. The number of colonies growing on the culture medium was counted after 4 days. Three kinds of 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/$CaCl_2$ method (Sun et al. 2015). First, the fungal protoplasts of 150 μl were placed on ice for 10 min. Then the protoplasts were added with 1μl of heparin solution (10 mg mL^{-1}), and 3-5 ug linearized plasmid, iced for 10min. Next, the STC-PEG (SPTG: 4g 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 d, and then transferred to the selective medium with sulfonylurea for another 3-5d. 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, 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

All the data were obtained in triplicate and statistically analyzed by SPSS 18.0. The significant differences from others conditions were at $P < 0.05$ level, and the values represented as the mean ± standard deviation.

Results

Screening of optimum conditions for isolation and regeneration of protoplasts

Digestion enzyme is a crutial factor controlling the efficiency of protoplast transformation. Three cell wall digestion enzymes (glucanase, lysing enzyme, and snailase) were tested alone or combined for releasing protoplasts from *F. oryzae*. The results showed that glucanase had a higher yield of protoplasts than the other two enzymes alone (Fig. 1A). Furthermore, the combination of enzymes was more efficient than a single enzyme. The combination of glucanase, lysing enzyme and snailase yielded the highest protoplasts, followed

by the combination of glucanase and snailase (Fig. 1A). However, considering the operability and cost, the use of glucanase alone can produce sufficient protoplasts for transformation.

The number of fungal protoplasts increased along with glucanase concentration and reached a maximum at 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).

Along with the increase of incubating time, the number of protoplasts released gradually increased. When incubated for 3 hours, the release of protoplasts reached a peak at 12.6×10^{7} g^{-1} (Fig. 1D). Prolonging incubation time did not increase the yield of protoplasts significantly. Prolonged incubation time damaged 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 is thickened with age, and protoplasts are challenging to be released. In turn, it is easily destroyed, and more protoplasts are released. 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 CM liquid medium to ensure that the hyphae were young and in logarithmic growth phages. The protoplast yield increased continuously in 1–3 days, keeping stable on the 4th day. 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 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 (Ma et al. 2014). The results also showed that the protoplast regeneration rate reached highest when RgA medium containing 1 M sucrose.

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

	Digestion solution				RgA medium	
Osmotic stabilizers	Concentration (mol L^{-1})	Protoplast yield (10^7 g^{-1})	Regeneration cells (10^6 g^{-1})	Protoplast regeneration rate (%)	Concentration (mol L^{-1})	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}**
	1	9.0 ± 0.26^{d}	4.73 ± 0.25^{d}	5.25 ± 0.92	1.2	16.77 ± 1.10^{fg}
NaCl	0.8	1.6 ± 0.26^{fgh}	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^{i}
	1	1.67 ± 0.32^{fg}	0.87 ± 0.06^{ef}	5.2 ± 0.34	1.2	3.53 ± 0.67^{j}
Sucrose	0.4	1.97 ± 0.29^{f}	0.93 ± 0.15^{ef}	4.74 ± 0.57	0.8	33.63 ± 2.58^{c}
	0.5	9.80 ± 0.89^{c}	11.03 ± 0.47^{b}	11.25 ± 0.85	1	42.2 ± 2.67^{a}
	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 means $P < 0.05$.

Antibiotic resistance assay and transformation of GFP-vector in F. oryzae

To insert exogenous DNA fragment into *F. 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 when the sulfonylurea concentration reached 300 μg

ml^{-1}, colony growth was completely inhibited (Fig. 2A, B), suggesting that the minimum inhibitory concentration (MIC) value for *F. oryzae* was 300 μg ml^{-1}. We thus used this antibiotic concentration for the selection of *F. oryzae* transformants generated from the protoplast transformation.

Fluorescence expression vector pKD6-GFP was transfected into the protoplasts of *F. oryzae*. First, the GFP-tagged transformants were grown on DCM medium supplemented with 1M sucrose and 300 μg mL^{-1} sulfonylurea for 5–7 days. Then the regenerated transformants were re-cultured on a DCM medium containing 300 μg 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 also suggested that exogenous promoter SOD1 promoter 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). And, 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, which facilitated the observation of mycelia 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 first reported and had a high transformation efficiency. To deeper 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 were proved to be of excellent efficiency in the protoplast system.

An effective transformation system is a prerequisite for studying fungal genetic manipulation and functional genomics (Dobrowolska and Staczek 2009). ATMT is widely used for transforming various fungal materials (Groot et al. 1998; Chen et al. 2000). In *F. oryzae*, ATMT 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, and 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 fresh fungal hyphae

digested with 10 mg mL^{-1} of glucanase in 4 mL 0.9 ml solution containing 0.9 M KCl for 4 h could generate approximately 4×10^8 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. Because the components of the fungal cell walls are highly complex and dynamic and vary among fungi species. Therefore, the selection of an appropriate enzyme and enzyme concentration is crucial 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 enzymes and are more easily degraded to remove the cell wall. The 4-hour-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 is 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 strains genes. This technique enables scientists to target and modify genes efficiently to reveal 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 (Martín 2015). The selection of appropriate promoters is also an essential factor for the success of genetic transformation. Here, the superoxide dismutase (SOD1) gene is highly expressed at various stages of fungal development. EGFP with SOD1 as the promoter was uniformly, firmly, and stably expressed in hyphae and conidia of *F. oryzae*, and had a robust activating effect in hyphae and conidia. During the symbiosis process of rice and *F. oryzae*, the fluorescence expression was still stable and coherent in the mycelium. Furthermore, the intensity of fluorescence expression was strong enough not to be covered by the spontaneous fluorescence from plant roots. Thus, these two advantages make SOD1 to be an excellent promoter choice.

Many functional genes can be studied in this way for subsequent molecular mechanisms and symbiotic processes. The demonstration is the most effective genetic transformation in *F. oryzae*. The described system also creates opportunities for future genetic research in other endophytic fungi.

Declarations

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Author contribution

Fu-Cheng Lin and Zhen-Zhu Su conceived and designed the experiments. Zhen-Zhu Su, Meng-Di Dai, Yu-lan Zeng performed the experiments. Jia-Nan Zhu, Xuan-jun Lu, and Xiao-hong Liu analyzed the data. Fu-Cheng Lin, Zhen-Zhu Su, and Meng-Di Dai wrote the manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

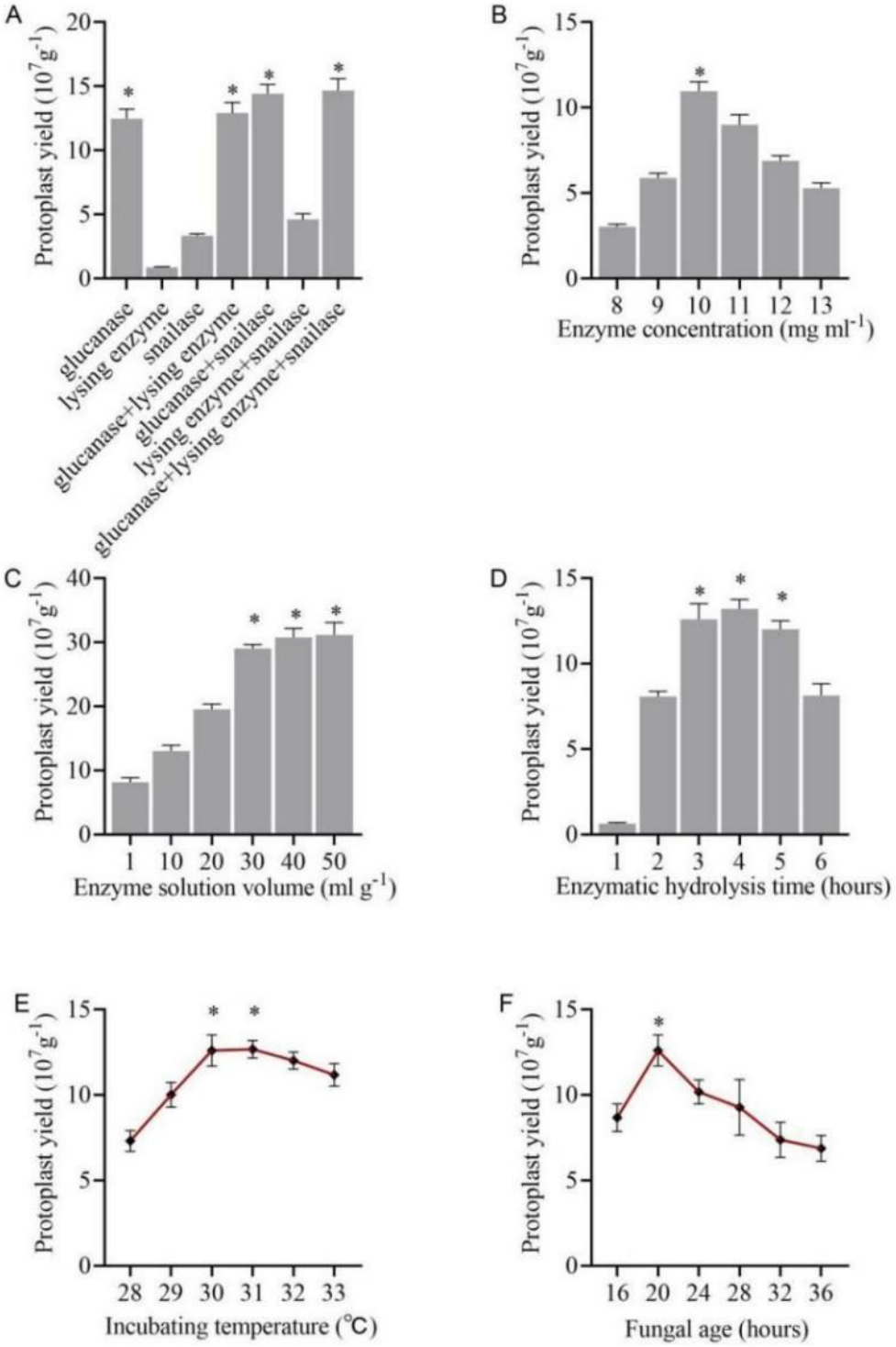

Figure 1

Effect of enzymes and biomass on protoplast formation. Three enzymes, glucanase, lysing enzyme, and snailase were tested alone or in combination for protoplast releasing from F. oryzae cells. Effects of enzyme combinations (A), enzyme concentration (B), enzyme solution volume (C), enzymatic hydrolysis time (D), incubating temperature (E) and fungal age (F) were evaluated for the formation of protoplasts. Y-axis indicates the number of protoplasts produced from 100 mg fungal with fresh weight, and protoplasts were measured by hemocytometer under a microscope. The data represent means, and error bars are standard deviations from three biological replicate samples. The bars represent the means±SDs. Significant differences (t test): * means $P<0.05$.

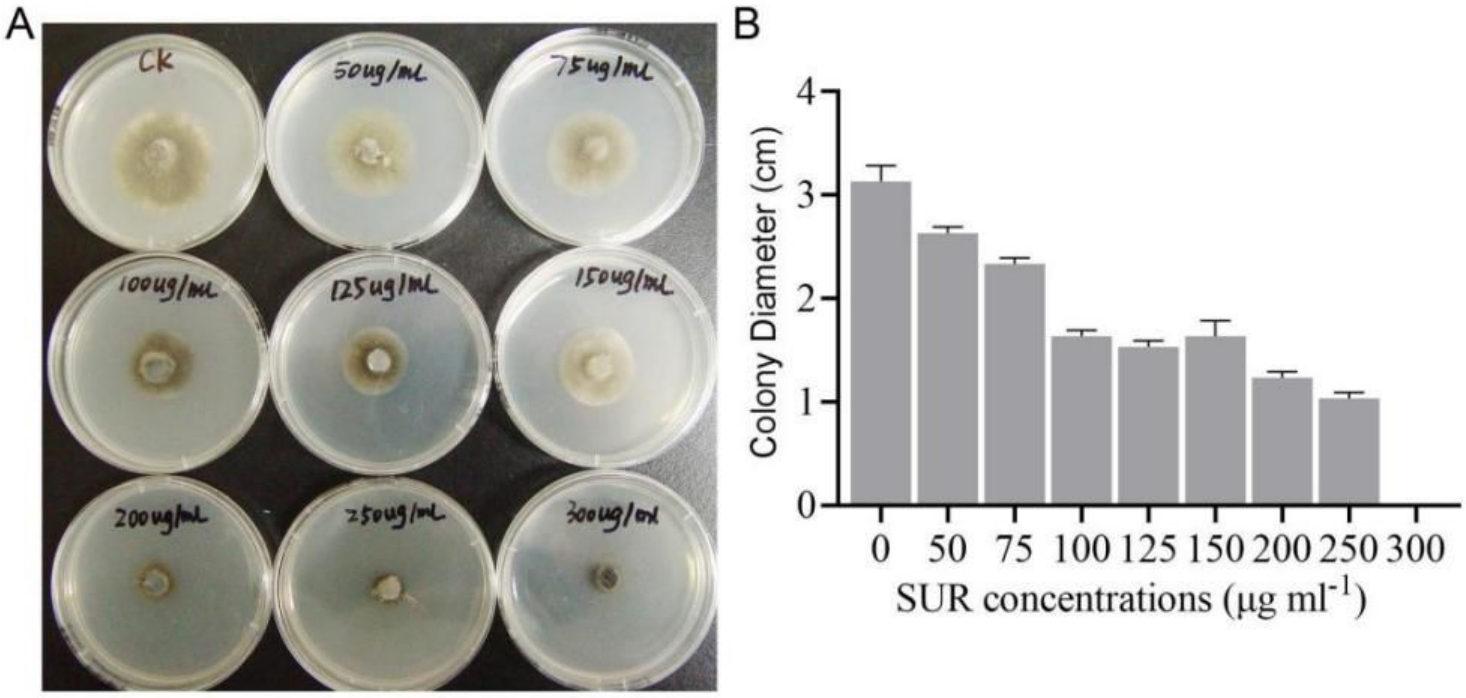

Figure 2

Sensitivity test to antibiotic in F. oryzae. (A) The mycelial growth of F. oryzae was analyzed on DCM with various concentrations of hygromycin after 7 days incubation before observation. (B) The colony diameter of F. oryzae under SUR. The bars represent the means±SDs, n=3.

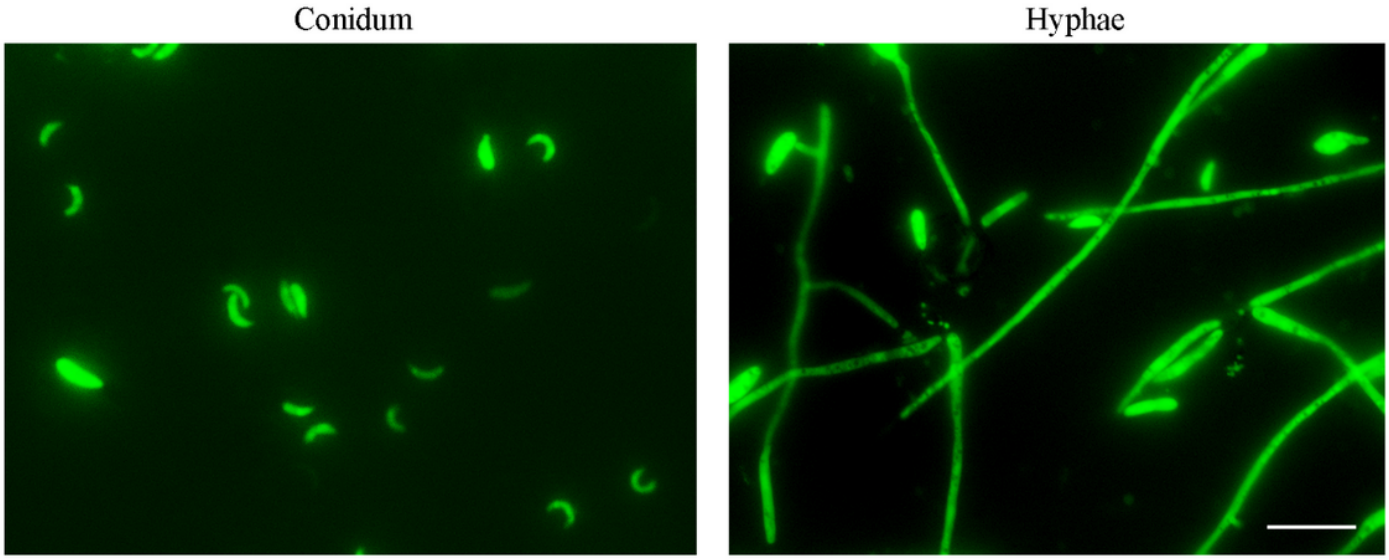

Figure 3

The fluorescence in hyphae and conidia from three generations of transformants pKD6-GFP. Scale bar=50 μm.

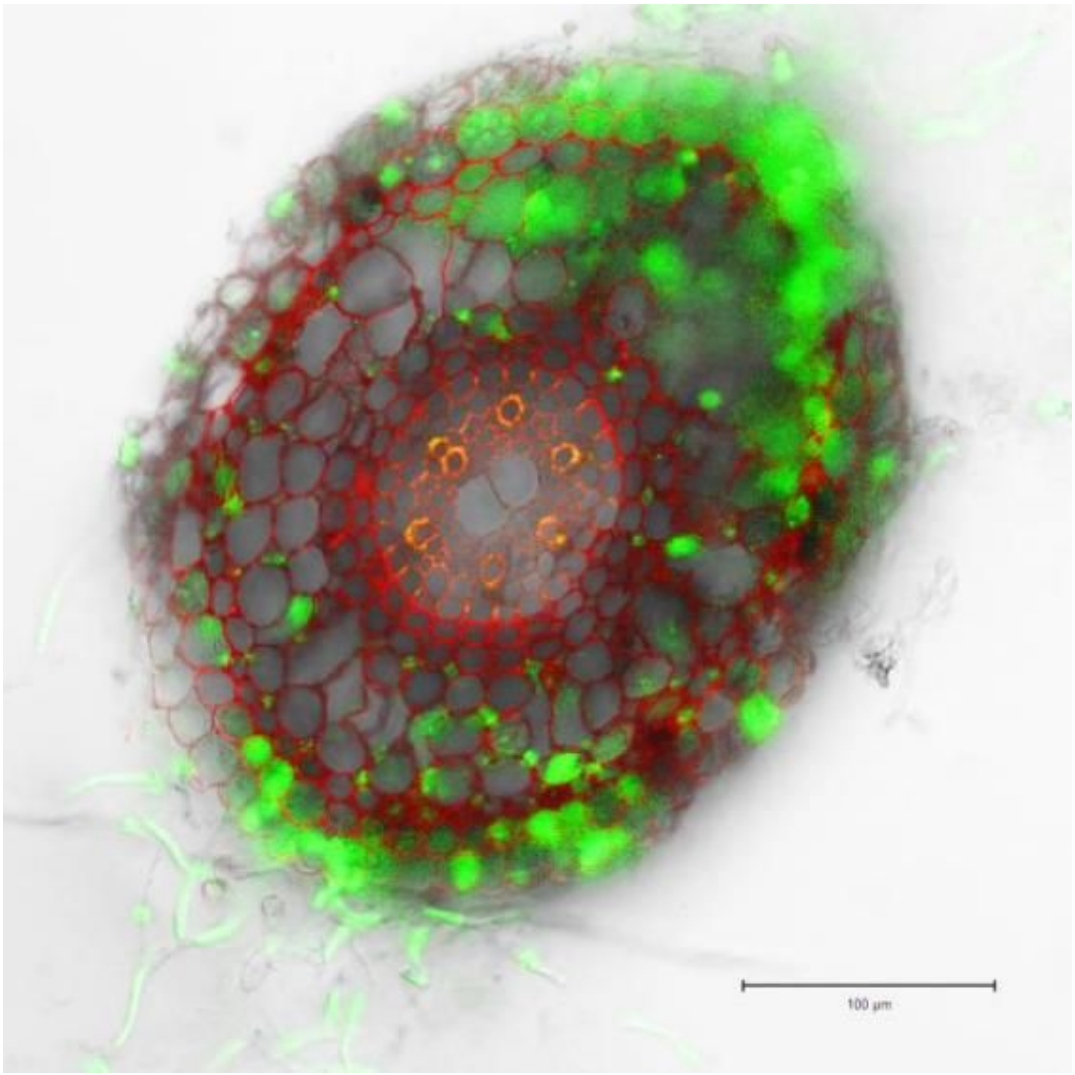

Figure 4

Colonization of F. oryzae in rice roots. In a root cross-section, GFP-tagged hyphae gradually extended from the epidermis to the cortex without penetrating the stele. Scale bar=100 μm.