

Full Length Research Paper

Development of a simplified and efficient method for genetic transformation of *Gymnoascus reesii*

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The identification of complex genetic determinants that control the ability of a fungus to produce bioactive compounds enhances molecular breeding strategies. Through a series of optimizations, we developed a system for the genetic transformation of *Gymnoascus reesii* that can produce nematocidal activity against the root-knot nematode *Meloidogyne incoginta*. The optimal conditions for protoplast preparation and generation are as follows: mycelia growth time in CM medium of 42 h, Driselase as the cell wall-degrading enzyme, a ratio of 1.5 mL enzyme solution (20 mg/mL Driselase) per gram mycelia, a treatment time of 4 h at 28°C, and 0.7 M NaCl buffer. With the above conditions, the yield of *G. reesii* protoplasts reached 1.84×10^7 protoplasts per mL with a regeneration rate of up to 21.17% on solid regeneration medium (SR medium). The pKNTG vector with green fluorescent protein (GFP) was transformed into the protoplasts by polyethylene glycol (PEG), and 20 positive transformants were obtained. The results provide a basic technique for molecular breeding of *G. reesii* strains that has improved nematocidal activity.

Key words: *Gymnoascus reesii*, nematocidal activity, genetic transformation.

INTRODUCTION

Gymnoascus reesii is a soil ascomycete and also a potential material for controlling fungal or nematode diseases in plants. Benjamin et al. (2005) reported that *G. reesii* culture filtrates displayed significant activity against the nematode *Haemonchus contortus* and significantly inhibited the growth of the bacterium *Bacillus subtilis*, the plant fungal pathogen *Septoria nodorum* and a tumor cell line (murine NS-1) (Benjamin et al., 2005, 2006). Liu et al. (2004) reported that they screened a *G. reesii* isolate named Za-130 that exhibited high nematocidal activity against *Meloidogyne hapla*; they also identified the nematocidal metabolite to be (3E, 5E)-2, 5-dihydroxy-2,7-dihydrooxepine - 3- carboxylic anhydride by bioassay-

guided fractionation (Liu et al., 2011).

Developing a well-defined and efficient system for the introduction of DNA into *G. reesii* will facilitate the cloning of *G. reesii* genes that control the production of bioactive compounds. Transformation systems for a number of model ascomycete species have been reported. These systems allow the transformation of *Saccharomyces cerevisiae* (Hinnen et al., 1978; Schiestl and Petes, 1991), *Neurospora crassa* (Case et al., 1979), *Aspergillus nidulans* (Yelton et al., 1984), *Magnaporthe oryzae* (Parsons et al., 1987) and others. With the whole genome sequencing of the above model fungi and efficient genetic transformation systems, gene knock-out and gene complementary systems have also been established in model ascomycete species (Colot et al., 2006; Hamer et al., 2001). For biocontrol materials, genetic transformation studies have been performed mainly in *Trichoderma harziznum*, *T. reesei* and *T. viride* (Penttila et al., 1987; Manczinger et al., 1997; Wang et

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al., 2009). Biocontrol-related genes have also been identified in *T. harzianum*, *Chaetomium cuperium* and *C. globosum* by expressed sequence tags (ESTs) (Yang et al., 2007). Good methods for genetic transformation are required for molecular breeding of fungi that produce bioactive compounds more efficiently.

Functional analyses of some biocontrol materials have been carried out in the last several years, and many functional genes were cloned, especially in *Trichoderma* (Montero-Barrientos et al., 2011; Pozo, 2004). However, the genetic transformation of *G. reesii* has not been reported, and no functional studies have been carried out in this species. Thus, the objective of this study was to create a simple and efficient method for the genetic transformation of *G. reesii* for future genetic engineering of strains that produce bioactive compounds.

MATERIALS AND METHODS

Chemicals

Driselase, casein enzymatic hydrolysate, casein acids hydrolysate, and CaCl_2 were products of Sigma. Sorbitol and Tris base were Amresco products, and yeast extract and tryptone were from OXOID Company (UK). The agar was bought from Takara Company (Dalian, China), and glucose, sucrose and other common reagents were purchased from Beijing Chemical Reagent Company (Beijing, China).

Strains and plasmids

G. reesii Za-130 (CGMCC No.2632) used in the present study was isolated by Liu et al. (2004). The isolate was maintained and cultured on potato dextrose agar (PDA) medium in the Biological Control Laboratory, Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences. The plasmid pKNTG containing neomycin phosphotransferase and GFP under the control of the RP27 promoter was provided by Dr. You-Liang Peng (State Key laboratory for Agrobiotechnology, China Agricultural University).

Medium

The normal medium PDA was made as follows: 200 g of potato was boiled for 20-30 min and then filtered with 4 layer gauze; 2% glucose and 1.5-2% agar were added, and ddH_2O was added to 1000 mL. Mycelium culture was the complete medium (CM, 0.6% yeast extract, 0.3% casein enzymatic hydrolysate, 0.3% casein acids hydrolysate, and 1% sucrose). The regeneration medium was liquid regeneration medium (LR: 0.1% yeast extract, 0.1% casein enzymatic hydrolysate, 1 M sucrose) and solid regeneration medium (SR: LR with 0.7% agar). The transformation reagents were STC solution (1.2 M sorbitol, 10 mM Tris-Cl, pH 7.5, 50 mM CaCl_2) and PTC solution (6% polyethylene glycol 3350, 10 mM Tris-Cl, pH 7.5, 50 mM CaCl_2).

Protoplast preparation

G. reesii isolate Za-130 cultured for 3-5 days on PDA medium in a Petri dish was rinsed with 2-3 mL sterile water, and then the mycelium was transferred to 500 mL flasks containing 150 mL CM

liquid medium by 1 mL tips. The mycelium was cultured at 28°C at 130 r/min. After 36, 42 and 48 h, the mycelium was collected with sterile 3 layer lens cleaning paper (Double loop Company, China) with quartet built filter funnel, and then the collected mycelium was fully washed with an osmotic stabilizer. Every 0.3-0.5 g mycelium was transferred to a 50 mL centrifuge tube. The Driselase (20 mg/mL, dissolved in 0.7 M NaCl) was added in the tube at a ratio of 1.5 mL enzyme solution per 1 g mycelium. The mycelium was lysed at 28°C, 120 r/min for 3 to 6 h. The protoplasts were collected with 3 layer lens cleaning paper, followed by centrifugation (4000 r/min, 4°C, 15 min), and then the protoplasts were resuspended with 1 mL STC. The number of protoplasts was calculated with a Blood Count Board (Shanghai Refinement Biochemical Instruments Co., LTD, China), and the number of protoplasts collected from 1 g mycelium lysed by Driselase was defined as the yield of protoplasts.

Protoplast regeneration

The protoplasts were centrifuged at 4°C and 2000 g for 15 min and then adjusted to 1×10^4 protoplasts/mL with osmotic stabilizer. Protoplasts (0.1 mL) were spread on the SR or PDA medium plates. Meanwhile, 0.1 mL protoplasts mixed with 1 mL sterile water were kept for 30 min under room temperature and then were spread on the SR or PDA plates as controls. The plates were placed at 28°C for 3 to 5 days until small colonies emerged on the flat surface. The experiment was conducted for 3 times. The rate of protoplast regeneration (%) = [(number of small colonies on regeneration medium - number of small colonies of CK) / number of protoplasts] $\times 100\%$.

Transformation mediated by PEG

The protoplasts were washed three times with 10 mL STC solution and then suspended to a final concentration of 1×10^8 protoplasts/mL with STC solution. Transformation was performed by incubating 150 μL protoplasts with 2-3 μg linear pKNTG plasmid DNA in a 50 mL centrifugation tube for 20 min on ice, and then 2 mL PTC solution was added. The tube was placed on ice for 20 min, and then 25 mL STC solution was added and the sample was centrifuged at 4°C, and 4000 r/min for 15 min. The pellet was resuspended with 3 mL LR, and then the mixture was put into the incubator at 28°C for 12 h. The mixture was added to SR to pour on plates and then covered with 0.7% top agar that contained 400 $\mu\text{g}/\text{mL}$ of neomycin. The plates were placed in the RXZ illuminating incubator (Ningbo Jiangnan Instrument Company, China) until small colonies were observed, with 12 hours of light cycle at 28°C.

DNA extraction

Genomic DNA was extracted from mycelia using the hexadecyl trimethyl ammonium bromide (CTAB) procedure described by Talbot et al. (1993). Plasmid DNA was isolated by the method of Noguchi (1991).

Identification of transformants

The GFP gene was amplified from the DNA extracted from transformants with GFP-F/R primers (GFP-F: 5'-ATGGTGAGCAAGGGCGAGG-3', GFP-R: 5'-TTACTTGTACAGCTCGTCC-3'). The polymerase chain reaction (PCR) mixture (25 μL) was as follows: 0.25 μL of rTaq DNA polymerase (5 U/L), 2.5 μL of $10\times$ buffer (plus Mg^{2+}), 1 μL of extracted genomic DNA, 0.5 μL of GFP-F primer (10 $\mu\text{mol}/\text{L}$), 0.5 μL of GFP-R primer (10 $\mu\text{mol}/\text{L}$), 2 μL of dNTP (each 2.5 mmol/L) and

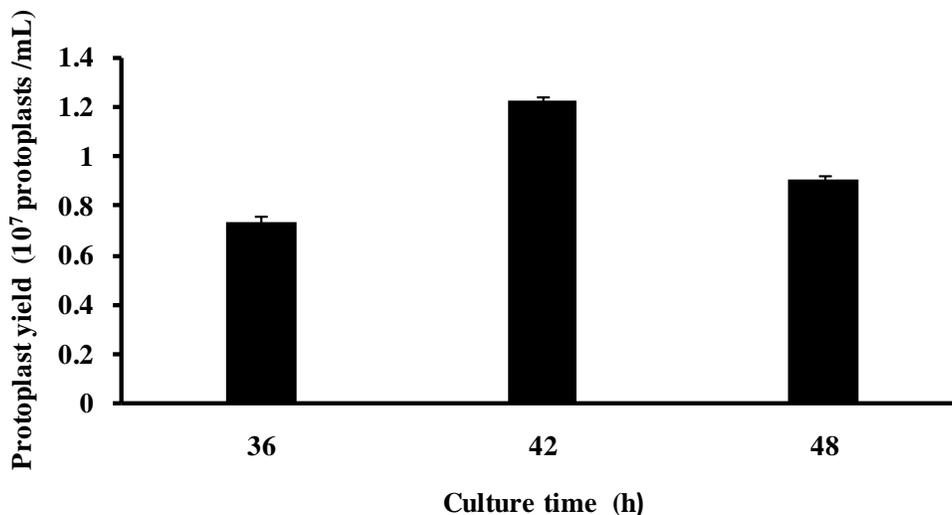


Figure 1. Effect of culture times on the protoplast yield.

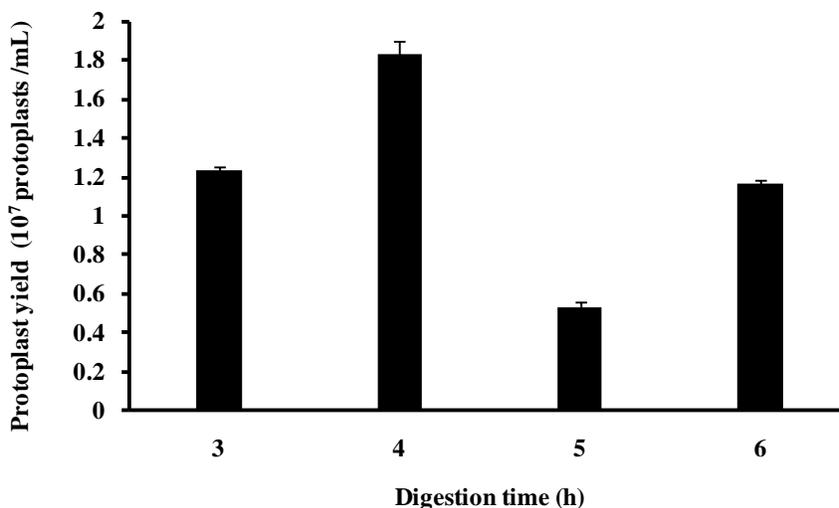


Figure 2. Effect of digestion time on the protoplast yield

18.25 μ L of deionized water. The PCR reaction conditions were as follows: 30 cycles of 95°C for 1 min; 56°C for 1 min; 2°C for 1 min, with a start of 95°C for 4 min and finish of 72°C for 10 min. PCR products were detected by 1.0% agarose gel electrophoresis.

Fluorescent hyphae were identified using a Nikon ECLIPSE 80i (Nikon, Japan) with UV light. The GFP signal was observed with a Nikon DS-Ri1 (Nikon, Japan), and images were analyzed by NIS-Element D (Nikon, Japan).

RESULTS

The optimal mycelium culture time

The mycelium was cultured for 36, 42, or 48 h and lysed. When the mycelium was cultured in medium for 42 h, the

yield of protoplasts reached a peak of 1.24×10^7 protoplasts/mL (Figure 1). These results suggest that the lysis of mycelium may correlate with the growth of the cell wall and that when the mycelium was cultured for 42 h, the conditions of the cell wall were suitable for lysis.

The optimal mycelium cell wall digestion time

The yield of protoplasts was compared after different digestion periods with Driselase, from 2 to 6 h. The results (Figure 2) indicated that the yield was highest at 4 h digestion. However, the difference between the yields at 3 and 6 h was not significant.

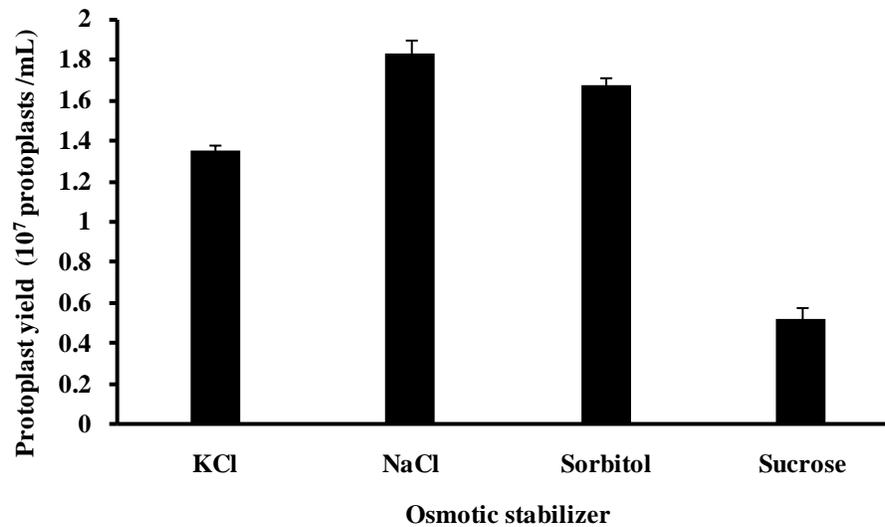


Figure 3. Effect of the osmotic stabilizer on the protoplast yield.

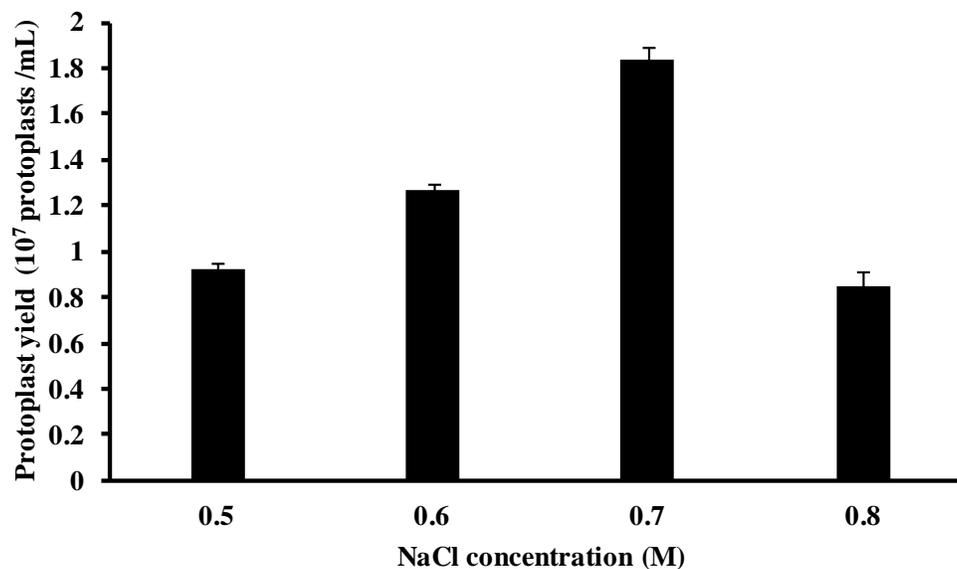


Figure 4. Effect of NaCl concentrations on the protoplast yield.

The optimal osmotic stabilizer for protoplast preparation

KCl, NaCl, sorbitol and sucrose solution were candidate osmotic stabilizers tested in this study (all solution concentrations were 0.7 M). The results (Figure 3) indicated that NaCl is the most suitable osmotic stabilizer for protoplast release from *G. reesii*. Different concentrations were tested to determine the optimal concentration, and the results shown in Figure 4 demonstrate that 0.7 M NaCl was the best osmotic

stabilizer.

The optimal regeneration medium for protoplast regeneration

PDA and SR medium were tested as candidate medium for regeneration. With a digestion time of 4 h, the regeneration rates of protoplasts on PDA and SR reached peaks of 19.21 and 21.17% (Figure 5), respectively. The differences between the two mediums were minor, but the

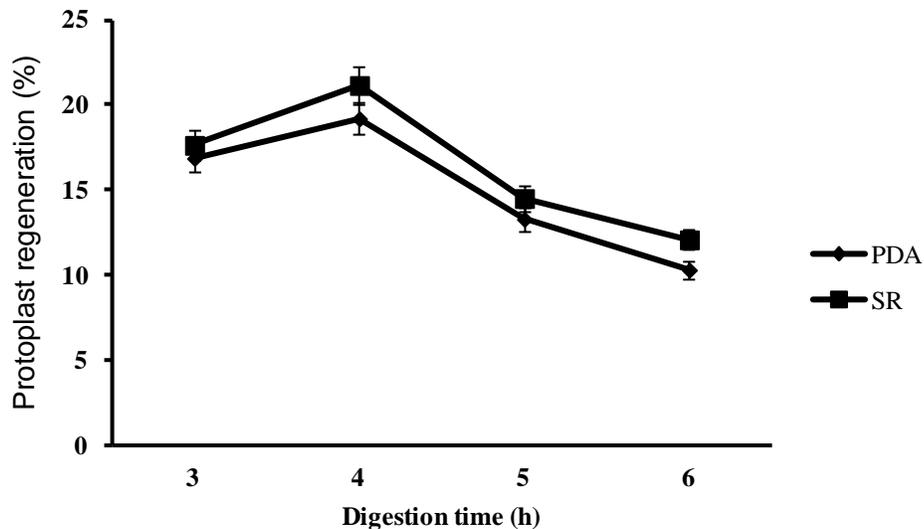


Figure 5. Effect of the medium and digestion time on the regeneration of protoplasts.

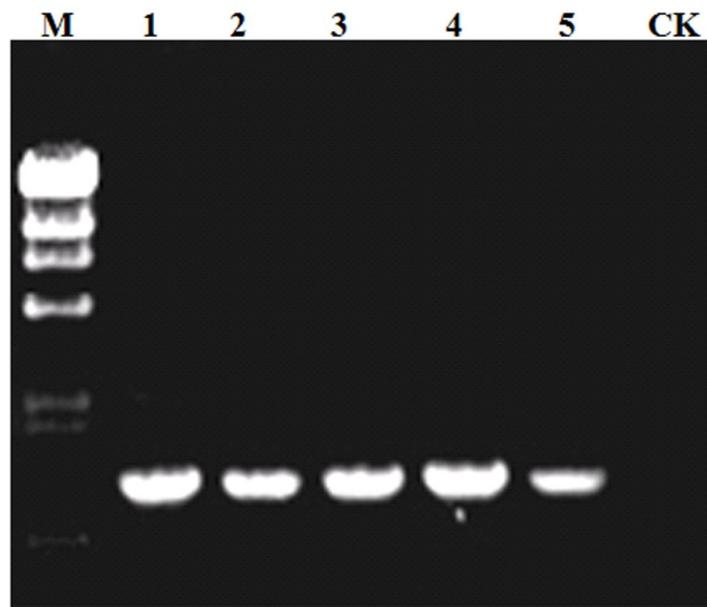


Figure 6. Confirmation of the integration of the plasmid pKNTG into transformant genomic DNA by PCR analysis with GFP-specific primers. M, λ DNA digested by *Hind*III; 1-5, five transformants randomly chosen; CK, Za-130 was used as a negative control.

SR medium was more suitable for these experimental conditions.

Identification of transformants by PCR and fluorescent microscopy

20 transformants of *G. reesii* were obtained by

transforming the linear pKNTG into wild type *G. reesii*. Ectopic integration of the construct was demonstrated by PCR amplification and GFP fluorescence. The 720 base pair nucleotide sequence of the GFP gene was amplified in all 20 resulting transformants as shown in Figure 6. The GFP from random positive transformants was detected in mycelia, whereas the control mycelia without transformation displayed no GFP (Figure 7).

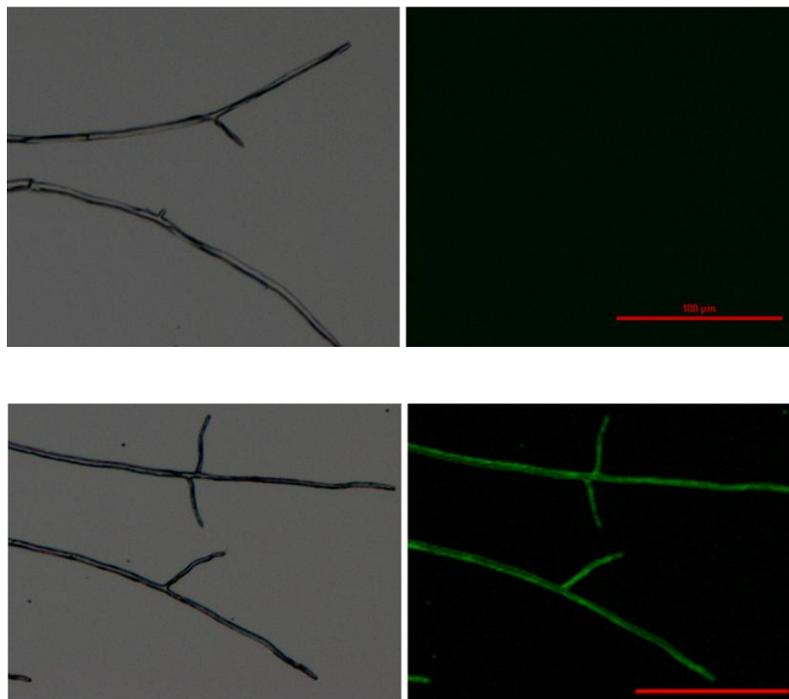


Figure 7. Expression of green fluorescent protein in *G. reesii* Hyphae of non-transformed Za-130 imaged under white light (A) and UV light (B). Hyphae of a transformant observed under white light (C) and UV light (D).

DISCUSSION

Plant disease caused by nematodes is a serious problem in the world, and fungal natural products are very promising potential sources of new chemicals to manage this (Anke and Sterner, 1997). Some biocontrol material-containing fungus commonly produce active compounds at low levels, and modification by molecular manipulation for efficient production of natural compounds or metabolic products is necessary. Protoplast preparation and regeneration is a key technology in molecular fungal research. PEG is a chemical that can alter the membrane of the fungal cell and allow exogenous DNA to enter into the cell. In the study, we used PEG and observed a high transformation efficiency. For the preparation of protoplasts, the type of culture medium, time of culture, and cell wall-degrading enzyme are the most important factors. The osmotic stabilizer not only affects protoplast release from the mycelium but also the activity of the enzyme, so a suitable osmotic stabilizer is also very important. The concentration of the osmotic stabilizer is another important factor that can affect protoplast release from *G. reesii*. When the concentration is high, the protoplast release will be inhibited, while low concentrations will cause protoplasts to expand and perhaps even burst. The digestion time and the type of regeneration medium can also affect the regeneration rate of protoplasts. In the study, we optimized each of

these factors with a series of experiments. The transformation system is stable and convenient, and we can get enough protoplast every time in our study.

Many functional genes have been isolated by insertional mutagenesis in the past few decades in different fungi (Sweigard et al., 1998; Bölker et al., 1995; Kang et al., 1994; Kuspa and Loomis 1992; Balhadere et al., 1999). We also simplified the Restriction Enzyme Mediated Integration (REMI) procedure so that daily transformation can be conveniently performed (results unpublished). At the same time, we also constructed the REMI transformation system, and got more than 2000 REMI transformants for gene functional analysis in future (data not shown).

The results reported in this paper have encouraged us to proceed with efforts to clone *G. reesii* functional genes that control or affect the production of compounds with bioactivity toward nematodes by transforming appropriate *G. reesii* recipients with suitable vectors. We have also constructed a library that contains 2016 REMI transformants that will be screened for isolates with more or less efficient production of nematicidal compounds.

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