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Promoter trapping in *Magnaporthe grisea*[*]

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Abstract: Application of promoter trapping based on transformation in *Magnaporthe grisea* is reported in this paper. Two promoter-trapping vectors, designated as pCBGFP and pEGFPHPH, were constructed and transformed into protoplasts of *M. grisea*. A library of 1077 transformants resistant to hygromycin B was generated. Of which, 448 transformants were found to express *eGFP* gene in different structures of *M. grisea.* Three transformants grew slowly, 5 transformants decreased in conidiation and 7 transformants reduced in pathogenicity greatly among these 448 transformants. Eleven transformants were checked by genomic southern blot randomly, and 9 of which were single-copy insertions. The promoter trapping technique has been applied successfully in *M. grisea* and can be used as a tool for functional genomic analysis.

Key words: Promoter trapping, Green fluorescent protein, *Magnaporthe grisea*
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INTRODUCTION

Magnaporthe grisea is a filamentous ascomycete that parasitizes economically important crops, such as barley, wheat, and rice (Talbot, 2003). It is also a good experimental model for studying fungal pathogenesis by both classical and molecular genetics (Dean, 1997). In recent years, many techniques have been developed to identify functional genes in *M. grisea* (Kamakura *et al*., 1999; Rauyaree *et al*., 2001; Takano *et al*., 2003; Irie *et al*., 2003; Lu *et al*., 2005). Of these techniques, insertional mutation method including REMI (restriction enzyme-mediated insertional mutagenesis) (Balhadère *et al*., 1999) and ATMT (*Agrobacterium tumefaciens*-mediated transformation) (Rho *et al*., 2001) is a powerful way for functional genomic analysis. This technique has been successfully used to transform diverse filamentous fungi and isolate important genes (Mullins and Kang, 2001) relying on screening a large number of transformants. Although it is an efficient tool for tagging and cloning functional genes from fungi, many tagged genes that have no obvious phenotype cannot be detected.

Promoter trapping, based on expression patterns of reporter gene, is one of the mutant techniques. This approach involves the introduction of a promoter-trapping vector containing a promoterless reporter gene into the genome, which leads to a result that expression can occur only when the insertion locates within a transcriptional unit and in the correct orientation. Expression of the reporter gene requires that it be inserted into an exon, leading to a transcriptional fusion (Springer, 2000). Recognizable phenotype is not indispensable, that is a remarkable advantage of the promoter trapping technique. This quality may enable us to study genes at earlier stage that are functionally redundant and have functions at multiple developmental stages, which are difficult to be ana-

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lyzed by conventional genetics.

Promoter trapping has been successfully applied to many organisms, including mammals (Gossler *et al*., 1989), insects (Bellen, 1999) and plants (Fobert *et al*., 1991). Furthermore, the potential benefit of this technique in understanding fungal genes has been realized. Several developmentally regulated genes have been demonstrated using the technique in *Dictyostelium discoideum* (Chang *et al*., 1995). The same procedure has also been applied in *Histoplasma capsulatum* (Retallack *et al*., 2000).

This paper describes a promoter trapping technology for *M. grisea*. Two vectors were constructed; both contain a promoterless reporter gene (*eGFP*) and the *hph* resistant gene. Library of promoter trapping transformants was established and the transformants have been characterized at different levels.

MATERIALS AND METHODS

Strains and culture conditions

M. grisea strain Guy11 was used as wild type and cultured in complete medium (CM) as described previously (Talbot *et al*., 1993). *Escherichia coli* strain DH5α was used as a host for plasmid amplification. The standard procedures were carried out (Sambrook *et al*., 1989).

Construction of the promoter trapping vectors

The vector pCBGFP was constructed on the backbone of pCB1003 (Carroll *et al*., 1994) and the portion of the plasmid pEGFP (Clontech, Palo Alto, CA, USA). The *Bam*HI-*Xba*I fragment of *eGFP* was separated from pEGFP and inserted into the *Bam*HI-*Xba*I sites of pCB1003 to generate pCBGFP (Fig.1a).

For construction of the pEGFPHPH, a 1.4 kb *hph* fragment with *Not*I and *Stu*I was amplified from the plasmid pCB1003 using the primers hph1 (5′-CCGCGGCCGCTGGAGGTCAACACATCAAT-3) and hph2 (5′-CCAGGCCTCTACTCTATTCCTTTGCCCTCG-3′) and cloned into the *Not*I-*Stu*I sites of pEGFP to create pEGFPHPH (Fig.1b).

The two vectors were verified by restriction digestion. pCBGFP vector was digested with *Bam*HI and *Xba*I, and a 700 bp fragment *eGFP* was produced. pEGFPHPH vector was digested with *Not*I and *Stu*I, and a 1.4 kb *hph* fragment formed. Restriction analysis showed that the promoter trapping vectors included the hygromycin-B gene and the promoterless *eGFP* gene, respectively. Vectors were subsequently linearized by *Bam*HI, *Kpn*I, *Sac*I, *Sma*I or *Age*I were used to transform protoplasts of *M. grisea*.

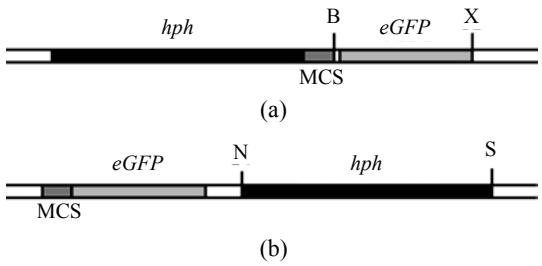

Fig.1 The skeleton of the two promoter-trapping vectors (a) Promoter-trapping vector pCBGFP, containing *Bam*HI-*Xba*I *eGFP* fragment cut from pEGFP; (b) Promoter-trapping vector pEGFPHPH, containing *Not*I-*Stu*I *hph* fragment amplified from pCB1003

MCS: Multiple cloning sites, including *Bam*HI, *Kpn*I, *Sac*I, *Sma*I, *Age*I restriction sites. The two vectors can be linearized by these restriction enzymes. *hph*: The resistant gene hygmycin B, 1.4 kb; *eGFP*: Reporter gene, 700 bp; B=*Bam*HI, X=*Xba*I, N=*Not*I, S=*Stu*I

Transformation of *M. grisea*

Protoplasts isolation was carried out as described by Talbot *et al*.(1993) with minor modifications. Briefly, a 3-cm^2 square of Guy11 mycelia was cut from the surface of the CM agar plate, incubated in 300 ml liquid CM medium at 28 °C on a rotary shaker at 125 r/min for 48 h. The mycelia were harvested by filtration and protoplasts were produced by Glucanex (Denmark) digestion in 0.7 mol/L NaCl. The protoplasts were washed in STC buffer (1.2 mol/L Sorbitol, 10 mmol/L Tris-HCl pH 7.5, 20 mmol/L $CaCl_2$) and adjusted to 10^8 protoplasts/ml. Transformation was performed using 150 μl protoplasts and 2 μg linearized pCBGFP and pEGFPHPH fragments, which had been digested respectively with different enzymes including *Bam*HI, *Kpn*I, *Sac*I, *Sma*I and *Age*I. Protoplasts were incubated with PTC buffer (60% PEG4000, 10 mmol/L Tris-HCl pH 7.5, 20 mmol/L $CaCl_2$) for 25 min and then 5 ml OCM (1.2 mol/L Sorbitol per 1 L CM liquid medium) was added to regenerate for 18 h at 28 °C. At this time, selective OCM agar medium containing 200 μg/ml hygromy-

cin B (Roche, Germany) was added. And the plates were incubated in the dark at 28 °C for 7 d. Monoconidial isolation was conducted on all hygromycin B-resistant transformants.

Fluorescence, morphological and cultural characteristics

Transformants purified by single spore isolation were transferred to 9-cm CM plates and kept for 7 d at 28 °C with a 12-h photophase to examine the morphology and cultural characteristics. Wild type Guy11 was used as control. Fluorescence of the transformants was observed using an Olympus-BX51 microscope with UV epifluorescence and appropriate filters.

Transformant growth was assessed by radial growth assay, and conidiation expressed as the number of conidia produced per milliliter of a plate culture brushed with 5 ml of water. Conidia of transformants harvested from 10-d-old mycelial cultures were filtered and resuspended to 10^5 conidia per milliliter in sterile distilled water. Droplets of conidial suspensions (20~50 ml) were placed on plastic cover slips and incubated under humid conditions at room temperature. Microscopic observations were made to study appressorium after 24 h.

***eGFP* specific PCR**

Fungal genomic DNA was extracted as described by Talbot *et al*.(1993). For confirmation of stable integration of the promoter trapping vector, 623 bp *eGFP* coding region was amplified using *eGFP* specific PCR and the genomic DNA of transformants as template. The *eGFP*-specific primers sequences were GFP1 (5′-TGGTGAGCAAGGGCGAGGAG-3′) and GFP2 (5′-CAGGGCGGACTGGGTGCTCA-3′).

The 25 μl PCR reaction cocktail contained 0.4 mmol/L of each deoxynucleoside triphosphates (dNTP), 25 μl 10×PCR buffer, 10^{-11} mol/L of each primer, 0.5 units Taq polymerase, and 2 ng of DNA. Reaction conditions were initial denaturation at 95°C for 2 min, denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, elongation at 72 °C for 45 s, for 35 cycles, and a final extension 72 °C for 10 min.

Southern blot

Total isolated DNA was digested with *Apa*I, then separated on a 0.7% agarose gel in 1×TBE buffer and blotted onto a positively charged nylon transfer membrane. Labelled probes (GFP PCR amplified product 623 bp) were allowed to blot DNA. Different sized fragments containing *eGFP* segments were hybridized as the marker and the positive control. Hybridization and washes were conducted under high stringency following the manufacturer's instructions for Digoxygenin high prime DNA labelling and detection starter kit I (Roche, Germany).

Pathogenicity test

Pathogenicity of some transformants was assessed with a rice CO-39 cut leaf assay (Balhadère *et al*., 1999). Leaf segments were excised from the second leaf of a 14-d-old rice seedling. Conidia suspensions were prepared from 10-d-old transformants grown on CM, adjusted to a final concentration of 1×10^5 conidia/ml. Then a 20 μl-droplet was deposited onto the upper side of the cut leaves maintained on 4% (*w*/*V*) distilled water agar plates. Disease lesions were observed after 4-d incubation at 28 °C with a 12-h photophase, with wild type Guy11 as control.

RESULTS AND DISCUSSION

Isolation of transformants expressing *eGFP* gene

After the transformation procedure, 1077 hygromycin B-resistant transformants of *M*. *grisea* were obtained. Among those transformants, 489 transformants fluoresced bright green. Strong constitutive expression of *eGFP* occurred and could be seen in the spores, mycelia, appressorium as well as both in the spores and mycelia (Fig.2). The number of various transformants is shown in Table 1. Expression of the *eGFP* gene under standard growth conditions was observed in 44% of our library. In 489 transformants fluoresced bright green, 57.67% transformants expressed in spore (Fig.2a), 38.45% in mycelium (Fig.2b), only 19 transformants in spore and mycelium (Fig.2d). In this study, strong expression of *eGFP* can be observed in the mature appressorium of transformants that the *eGFP* could be expressed in spore (Fig.2f). In the mean time, the expression of the *eGFP* gene was difference from transformants to transformants. The variation might be explained by the integration of the tag gene into different chromosomal sites. Screening different transformants can

be designed to identify genes that are expressed in specific structures or at specific stages.

Detection of the *eGFP* gene in transformants

The presence of *eGFP* gene in the transformants was confirmed with *eGFP* special PCR described above. The 623 bp PCR product was consistently amplified in the transformants fluorescing green, but the wild type was negative after the amplification reaction (Fig.3). The result confirmed that the *eGFP* fragment integrated the genome.

Analysis of insertion copies of transformants

In this paper, eleven transformants were randomly selected to check the copy number of the library. Successful hybridization occurred using the labelled *eGFP* probe but *eGFP* gene could not be detected in the wild type Guy11. Eleven transformants were examined randomly, and 9 of them were single-copy insertion (Fig.4). This result showed that about 4/5 transformants are single-copy insertion in our library. At the same time, the integration of GFP in the transformants were confirmed with Southern blot analysis.

A powerful asset for functional genomic analysis is the ability to create large annotated single gene mutant collections. It is prone to isolate and identify the genes in the low copy number transformants. Thus, the copy number is an important target to weigh the quality of the library after tag fragments integrated into the genome randomly. In our study, most transformants are single copy insertion in the promoter-trapping library. It is similar to the result of ATMT library described as Rho *et al*.(2001). The results showed that we could get valuable transformants easily in our library.

Growth and sporulation of the transformants

Analysis of the growth and the sporulation of 489 transformants among which the *eGFP* could be successfully expressed, showed that three transformants grew slowly (Fig.5a), and five transformants decreased in conidiation (Fig.5b). It was proposed that the insertion of the tag gene influenced the vegetable growth and sporulation.

Pathogenicity assays

Primary "rapid" assay on the cut leaves efficiently screened the pathogenicity of valuable transformants in the transformant library. In our study, we use this rapid assay to test the pathogenicity ability of 489 transformants. The deceased pathogenicity of seven transformants, *Bam*HI-52, *Bam*HI-31, *Bam*HI-101, *Bam*HI-104, *Sac*I-6, *Sac*I-31 and *Sac*I-61, was illustrated (Fig.6). The lesions of the transformants to leaves of rice seedling were smaller and couldn't spread any more after 4 d, compared with that of the wild type Guy11.

CONCLUSION

In conclusion, promoter-trapping depending on transformation was introduced successfully in *M. grisea*; two novel promoter-trapping vectors were constructed for transformation in this work. Those vectors contained the *hph* gene, encoding hygromycin B phosphotransferase, a commonly used selectable marker for transformation of fungal protoplasts and the *eGFP* reporter gene. By means of vectors, the *eGFP* gene can be used to analyze the strength of fungal promoters and to observe visually spatial and temporal expression patterns of fungal genes. Subsequently, sequencing the ends of these fragments and locating their genomic origin using available genome sequence data will identify the valuable promoters of functional genes. The novel genes in *M. grisea* will be expected to study further by this technique. In this work, transformants of promoter trapping in *M. grisea* fluoresced in different structures and influenced

Table 1 The number of the transformants and the site of *eGFP* expression

Restriction enzyme used	Total number of the transformants	Expression structures of the *eGFP* gene		
		Spores	Mycelia	Spores and mycelia
*Age*I	260	21	60	1
*Sac*I	190	56	21	5
*Sma*I	208	82	46	3
*Kpn*I	135	25	35	1
*Bam*HI	284	98	26	9

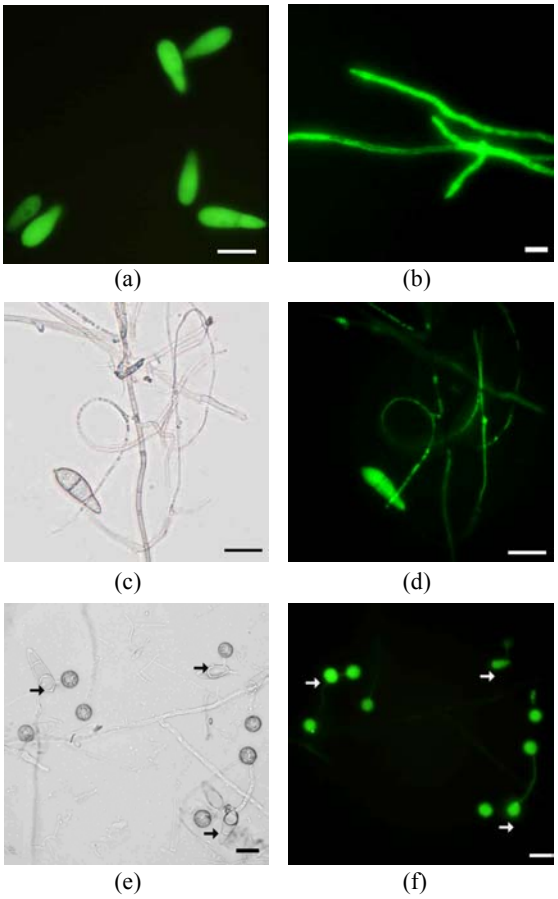

Fig.2 The expression of *eGFP* in the structures of transformants of *M. grisea* (a) Typical fluorescing spores; (b) Mycelium fluorescing bright green; (c) The bright field photo of the *eGFP* gene can be expressed in both the spore and mycelium; (d) The *eGFP* gene expressed in both spore and mycelium; (e) The bright field photo of the *eGFP* gene can be expressed in the 24 h-appressorium; (f) The 24 h-appressorium fluorescing bright green, and the arrows represent the spores (Bars=10 μm)

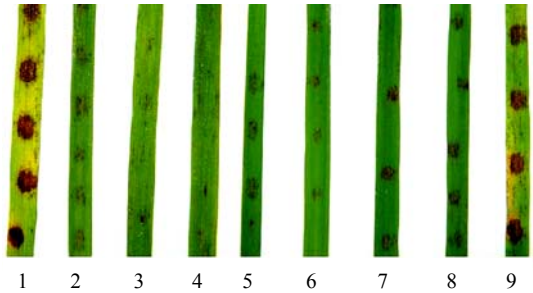

Fig.6 Results of pathogenicity test of the transformants
Seedlings from 14-d-old rice cv CO-39 were inoculated with 10^5/ml conidia from the wild type and transformants. Lanes 1 and 9 are wild type Guy11; Lanes 2~8 are transformants reduced in pathogenicity including *Bam*HI-52, *Bam*HI-31, *Bam*HI-101, *Bam*HI-104, *Sac*I-6, *Sac*I-31 and *Sac*I-61. Photos were taken 4 d after inoculation

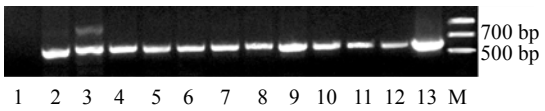

Fig.3 *eGFP* cassettes from transformants of *M. grisea* by PCR amplification
Lane 1: Negative control Guy11; Lanes 2~12: Amplified product using the transformants as template; Lane 13: Plasmid pEGFP as the template; M: 2 kb marker

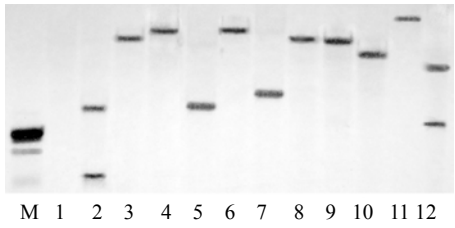

Fig.4 Copy number of *eGFP* insertion of some transformants in *M. grisea*
M: The fragments containing *eGFP* segment (*eGFP* PCR product, linearized pEGFP and pEGFPHPH); Lane 1: The wild type Guy11; Lanes 2~12: Selected transformants randomly; Lane 2 and Lane 12 are not single copy; others are single copy

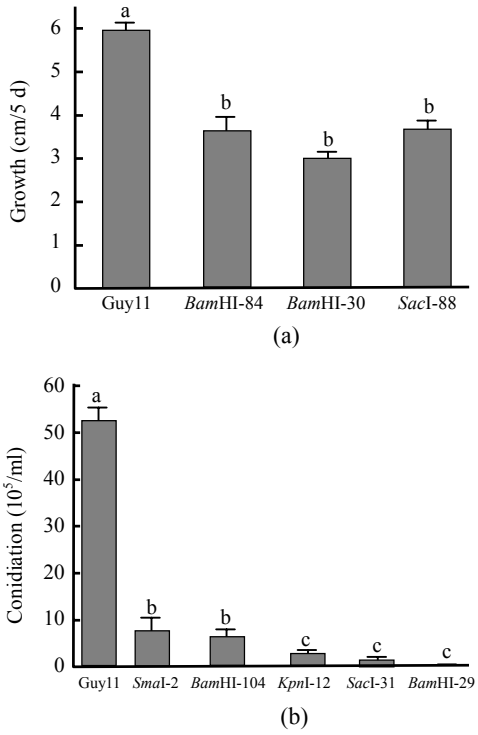

Fig.5 The growth and the conidiation of the transformants (a) The growth of the transformants which grew slower than the wild type Guy11; (b) The conidiation of the transformants which decreased compared with the wild type Guy11
Data presented as the average of five plates per treatment. Error bars indicate standard errors. Significant at P=0.01. a, b, c mean the significance

the aspects of growth, conidiation and pathogenicity to a considerable extent. Those were all valuable material to study the functional genes.

The efficiency and flexibility of the promoter trapping technique, makes it an efficient alternative to other techniques in characterizing important *M. grisea* genes. Therefore, promoter-trapping technique will be suitable as a complementary approach to full coverage of the *M. grisea* genome.

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