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生物技术与方法

GUS 为负标记的稻瘟病菌目标基因替换突变体双筛选体系

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摘　要: 目标基因替换是基因功能研究的重要方法, 在生物工程领域广泛应用。为了提高真菌目标基因替换的效率, 以稻瘟病菌为研究对象, 建立了一种以 *gusA* 基因为负筛选标记的目标基因替换突变体双筛选体系(GUS-DS)。首先, 检测了 78 个真菌菌株的内源 GUS 活性, 发现除 3 个菌株外均呈阴性。同时, 将 *gusA* 基因导入稻瘟病菌、镰刀病菌、炭疽病菌后, 转化子可获得高的 GUS 活性。表明 *gusA* 可用作真菌中的筛选标记。然后, 以 *gusA* 为负标记, *HPH* 为正标记, 以过氧化物酶体定位信号受体基因 *MGPEX5* 与 *MGPEX7* 的替换为例, 建立稻瘟病菌 GUS-DS 体系。对潮霉素抗性筛选获得的转化子通过 GUS 活性检测进一步筛选, 呈阴性者为可能突变体。通过 PCR 与 Southern 杂交对可能突变体进行验证, 以此评估 GUS-DS 的筛选效率。结果表明 GUS-DS 将 Δ*mgpex5* 与 Δ*mgpex7* 的筛选效率由原来的 65.8%和 31.2%分别提高到 90.6%和 82.8%。另外, 还建立了一种适合于 GUS-DS 的多重 PCR 法(M-PCR)用于突变体的验证。通过扩增目标位点的不同区段, 可以有效区分突变体、野生型和随机插入转化子。M-PCR 法验证突变体简便、迅速, 可信度与 Southern 杂交相同。GUS-DS 及 M-PCR 为功能基因组学及生物工程的研究提供了有力的工具。

关键词: 双筛选, GUS 活性, 目标基因替换, *MGPEX5*, *MGPEX7*

Dual screening for targeted gene replacement mutant in *Magnaporthe oryzae* with GUS as negative marker

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Abstract: To improve the efficiency of targeted gene replacement (TGR), a dual screen (DS) system with *gusA* gene as negative selective marker (GUS-DS) was developed in *Magnaporthe oryzae*. First, we tested the endogenous β-glucuronidase (GUS) activities of 78 fungal strains. All tested strains were GUS^-, only with 3 exceptions. Whereas, after the *gusA* being introduced in, *M. oryzae*, *Fusarium oxysporum* and *Colletotrichum lagenarium* acquired high GUS activities. The *gusA* is thus usable as a selective maker in fungal species. With *gusA* as the negative marker, *HPH* gene as the positive marker, and the peroxisomal targeting signal receptor genes *MGPEX5* and *MGPEX7* as 2 instances of target genes, we established the GUS-DS system. After transformation, we collected the transformants from hygromycin B screen media and then tested the GUS activities of them. The GUS^- ones were selected as potential mutants and checked in succession by PCR and Southern blotting to identify the true mutants and calculate the efficiency of

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GUS-DS. As a result, GUS-DS improved the screen efficiency for Δ*mgpex5* from 65.8% to 90.6%, and for Δ*mgpex7* from 31.2% to 82.8%. In addition, we established a multiple PCR (M-PCR) method for mutant confirmation. By amplifying the different regions at the targeted locus, M-PCR differentiated the wild type, the ectopic transformants and the mutants effectively and rapidly, and had the same reliability as Southern blotting. In conclusion, GUS-DS and M-PCR are useful tools to improve the efficiency of TGR and would be helpful for fungal genomics.

Keywords: dual screen, GUS activity, targeted gene replacement, *MGPEX5*, *MGPEX7*

Introduction

Generation of mutants by targeted gene replacement (TGR) is a well-established procedure for gene function analysis in many organisms [1–4]. In recent years, this procedure has been used to identify and characterize the genes in filamentous fungi, a group containing many plant pathogens and economically important species [5–8]. TGR is achieved via homologous recombination, where the target fungus transformed with a replacement vector yields two kinds of transformants: mutants and ectopic transformants. The ratio of mutants to ectopic transformants in filamentous fungi depends on the length of the homologous sequence, the extent of homology, the transformation method and the genomic position of the target gene [9, 10]. The ratio is often low, even in the cases with long homologous sequences, so we have to screen large numbers of transformants to find the mutants.

Positive and negative dual screen (DS) is an efficiency strategy to enrich targeted events. In DS systems, a negative selection marker is inserted into the gene replacement vector, flanking the replacement cassette which contains a positive marker. The positive marker is used to collect the transformants. And the negative marker can counter-select the ectopic transformants and enrich the targeted mutants. So far, DS systems have been used in filamentous fungi. And the negative markers used in the fungal DS include the herpes simplex virus thymidine kinase gene (*HSVtk*), the *oliC31* gene, and the *amdS* gene [11–14]. The β-glucuronidase (GUS) gene *gusA* is a widely-used gene marker in plants [15], whereas seldom reported in filamentous fungi [16]. To provide more agent as negative markers for DS and improve the efficiency of TGR in fungi, by using *gusA* as the negative marker and *HPH* as the positive marker, a GUS-mediated dual screen system (GUS-DS) in the rice blast fungus *Magnaporthe oryzae* was established. In addition, a convenient mutant confirmation method suited to GUS-DS, the multiple PCR (M-PCR) method, was introduced as well.

1 Materials and methods

1.1 Strains, media, and fungal transformation

M. oryzae strain Guy11 [17] was the wild-type strain for *gusA* transformation and gene replacement. *F. oxysporum* strain Fo0706 and *C. lagenarium* strain Cl0601 were the wild-type strains for *gusA* transformation. *Escherichia coli* strain DH5α was used in the cloning procedures. *A. tumefaciens* strain AGL1 was used in *Agrobacterium tumefaciens*-mediated transformation (ATMT).

The *M. oryzae* strains were cultured routinely on complete medium (CM) [18]. And other fungal species were cultured on potato dextrose agar (PDA) medium. *E. coli* and *A. tumefaciens* were cultured on Luria-Bertani (LB) medium. The inducible medium (IM) was prepared as described protocol [19]. The lipid-utilizing ability of mutants was tested on fatty acid medium (FAM; minimal medium agar with 0.5% (*V/V*) Tween 80) [7].

ATMT of *M. oryzae*, *F. oxysporum and C. lagenarium* were processed as the described procedures [19–21]. CM plates containing 250 μg/mL hygromycin B were used to screen *M. oryzae* transformants. PDA plates containing hygromycin B were used to screen transformants of other fungi (50 μg/mL for *F. oxysporum* and 200 μg/mL for *C. lagenarium*).

1.2 Vector construction

A 1.8 kb fragment of the *gusA* open reading frame (ORF) containing 5′ *Hin*d III and 3′ *Pst* I was amplified from pMDC140 [22] with primers GUSF-Hd and GUSR-Ps, and cloned into pBluescript SK$^+$ (Stratagene, USA) by digestion with *Hin*d III-*Pst* I to generate pBS-GUS. A 0.6 kb fragment of *Aspergillus nidulans trpC* terminator (TtrpC) containing 5′ *Pst* I and 3′ *Xba* I was amplified from pSH75 [23] with primers TtrpCF-Ps and TtrpCR-Xb, and cloned into pBS-GUS by *Pst* I-*Xba* I digestion to generate pBS- GUS1. The binary vector pCAMBIA1301 (Cambia, Australia) was digested with *Xho* I to remove the hygromycin B resistance gene (*HPH*) ORF and 2 unwanted *Pvu* I sites, and self-ligated to generate p1301X. Then a 2.4 kb fragment of *gusA* ORF followed with TtrpC containing 5′ *Hin*d III and 3′

Pvu I was amplified from pBS-GUS1 with primers GUSF-Hd and TtrpcR-Pv, and cloned into the *Hin*d III-*Pvu* I site of p1301X to generate p1301XG. A 0.7 kb fragment of *A. nidulans gpdA* promoter (PgpdA) containing 5′ *Xba* I and 3′ *Hin*d III was amplified from pBARGPE1 [24] with primers gpdF-CX and gpdR-Hd, and cloned into the *Xba* I-*Hin*d III site of p1301XG to generate p1301XGG. A 1.4 kb fragment of *HPH* under *A. nidulans trpC* promoter (PtrpC) containing *Bam*H I and *Sac* I was amplified from pCB1003 [25] with primer HPHF-Bm and HPHR-Sc, and cloned into p1301XGG by *Bam*H I-*Sac* I digestion to generate p1301XGGH.

The peroxisomal targeting signal (PTS) receptor gene *PEX5* (PTS1 receptor) ortholog (MGG_ 10840.5, assigned as *MGPEX5*, GenBank Accession No. EAA47029) and *PEX7* (PTS2 receptor) ortholog (MGG_01481.5, assigned as *MGPEX7*, GenBank Accession No. EAA55830) in *M. oryzae* were used to evaluate the efficiency of GUS-DS. To construct the replacement vector of *MGPEX5*, 1.4 kb 5′ flank and 1.8 kb 3′ flank sequences containing the appropriate restriction sites were amplified from the *M. oryzae* Guy11 genome with primer pairs prepex55-Xb/ prepex53-Sp, and postpex552-Ap/postpex53-Kn, respectively. In the same way, 0.9 kb 5′ flank and 1.0 kb 3′ flank sequences of *MGPEX7* were amplified with primer pairs UpPex75-Kn/Uppex73-Ap and Lowpex75-Xb/ Lowpex73-Sc. A 1.4 kb fragment of *HPH* under control of PtrpC containing *Bam*H I and *Sal* I was amplified from pCB1003. The 5′ flank, *HPH* and 3′ flank were cloned into multiple clone sites of pBluescript SK^+ in the correct orientation to generate pBS-pex5 and pBS-pex7. The replacement cassettes of *MGPEX5* and *MGPEX7* were cut from pBS-pex5 and pBS-pex7, respectively, and cloned into p1301XGG, to generate p1301XGG-PEX5 and p1301XGG-PEX7. The binary vectors p1301XGGH, p1301XGG-PEX5 and p1301XGG-PEX7 were used to transform *M. oryzae* by ATMT (Fig. 1). The primers used in vector construction are given in Table 1.

1.3 GUS assay

M. oryzae strains were cultured in 1.5 mL of CM liquid on 24-well cell culture plates, 1 strain per well, and shaken at 200 r/min at 27°C in darkness for 2 d. Then color substrate (0.5 mg/mL X-Gluc·Na) was added to the mycelium cultures. The plates were then transferred into 37°C darkness for 24 h to develop the blue color. Other fungal strains were treated using similar procedures in potato dextrose broth (PDB) media.

表 1 载体构建过程中所用的引物

Table 1 Primers used in vector construction*

Name	Sequences (5′–3′)
GUSF-Hd	CTAAGCTTATGTTACGTCCTGTAGAAAC
GUSR-Ps	ACCTGCAGTCATTGTTTGCCTCCCTGCT
TtrpCF-Ps	CCCTGCAGTGATTTAATAGCTCCATGTC
TtrpCR-Xb	CCTCTAGAAAGAAGGATTACCTCTA
TtrpcR-Pv	TCCGATCGAAAGAAGGATTACCTCTA
GpdF-CX	TGTCTAGATCGATTGCGGAGAGACGGACGGACG
GpdR-Hd	ACAAGCTTAGGGGAAATAAAGGTTC
TrpCF-CX	TG TCTAGATCGATGTTAACGTCGAGAGAAGATG
TrpCR-Hd	ATAAGCTTGGCTTGGGTAGAATAGGTAA
Prepex55-Xb	CTGTTGTCACGGTTCTAGAGGA
Prepex53-Sp	GACACTAGTTGTCGGTTGATGG
Postpex552-Ap	CGAGAGGGCCCGCGAGATCCTG
Postpex53-Kn	TGCGGTACCAGAAGCAGACATA
UpPex75-Kn	GGGGTACCAAGTCTGGCCCAGATTTAGTATTCA
Uppex73-Ap	CTGGGCCCGGAACTCAAGCATAGGAGCACTCAT
Lowpex75-Xb	GTTCTAGACACGACTTGACCAGCCTTACACGAT
Lowpex73-Sc	GGGAGCTCTGCACACTGGGTAGCGCGGAACGAC

*Restriction sites are underlined.

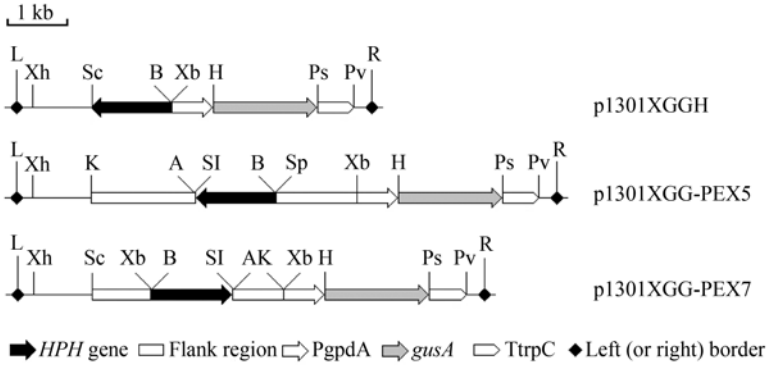


图 1 载体 p1301XGGH、p1301XGG-PEX5 和 p1301XGG-PEX7 的结构

Fig. 1 Structures of p1301XGGH, p1301XGG-PEX5 and p1301XGG-PEX7. L: left border; R: right border. Restriction sites: A: *Apa* I; B: *Bam*H I; H: *Hin*d III; K: *Kpn* I; Ps: *Pst* I; Pv: *Pvu* I; Sl: *Sal* I; Sc: *Sac* I; Sp: *Spe* I; Xb: *Xba* I; Xh: *Xho* I.

1.4 Dual screening and disrupting confirmation

The potential mutants bearing hygromycin B resistance and negative in blue development were checked by M-PCR and Southern blotting. The primers and probes used in M-PCR and Southern blotting are given in the results. Lipid utilization by the mutants of *MGPEX5* and *MGPEX7* were tested on FAM plates.

1.5 Nucleotide acid manipulation

Genomic DNA was extracted from fungal mycelium using a CTAB (Hexadecyltrimethylammonium bromide) procedure [26]. PCR, restriction digestion, gel electrophoresis and ligation reaction were all carried out using standard procedures [27]. For DNA gel blot hybridization, genomic DNAs from different strains were digested, size separated on a 0.7% agarose, transferred to a nylon membrane. Probes labeling and DNA hybridization were processed by using the DIG high prime DNA labeling and detection starter kit I (Roche, Germany).

2 Results

2.1 GUS activity in fungal strains and gusA transformants

To assess the possibility of using *gusA* gene in fungi, we first detected the GUS activity of different wild strains. The endogenous GUS activities in 78 wild fungal strains of about 20 fungal species were tested using the GUS assay (Table 2). A *Penicillium* sp. strain P0704 from air showing GUS activity at a high level was used as the positive control. After 24 h incubation, the mycelium of P0704 turned bright blue, another 2 strains (*Colletotrichum* sp. strain Cds0701, *Curvularia* sp. strain Cur0503) turned weak blue, and all the rest strains with unchanged color were regard as GUS^- strains.

表 2 不同真菌菌株的种类、寄主、症状与 GUS 活性

Table 2 Species, hosts, symptom and GUS activity in fungal strains

Fungal species	Nos[a]	Hosts	Symptom	GUS[b]
Alternaria alternata	6	Thunberg Fritillary (*Fritillaria thunbergii*)	Black spot	–
A. solani	5	Tomato (*Lycopersicum esculentum*)	Early blight	–
Botrytis cinerea	5	Cucumber (*Cucumis sativus*)	Gray mold	–
Cercospora sp.	3	Japan Hop (*Humulus scandens*)	Leaf wilt	–
Colletotrichum graminicola	3	Large Crabgrass (*Digitaria sanguinalis*)	Leaf spot	–
C. lagenarium	4	Watermelon (*Citrullus vulgaris*)	Anthracnose	–
C. lagenarium	3	Pumpkin *(Cucurbita moschata)*	Anthracnose	–
C. sp.[c]	2	Job's-tears (*Coix lacrymajobi*)	Leaf spot	–
C. sp.[d]	2	Large Crabgrass (*Digitaria sanguinalis*)	Leaf spot	+
Curvularia sp.	3	Large Crabgrass (*D. sanguinalis)*	Leaf spot	+
F. oxysporum f. sp. *niveum*	3	Watermelon (*Citrullus vulgaris*)	Fusarium wilt	–
F. oxysporum f. sp. *cucumerimum*	3	Cucumber (*Cucumis sativus*)	Fusarium wilt	–
F. oxysporum f. sp. *carthami*	3	Safflower (*Carthmus tinctorius*)	Fusarium wilt	–
Gibbrella zeae	3	Wheat (*Triticum aestivum*)	Fusarium head blight	–
Magnaporthe oryzae	8	Rice (*Oryza sativa*)	Rice blast	–
Penicillium italicum	2	*Citrus* (*Citrus sinensis*)	Green mold	–
P. sp.[e]	1	Air		+
Phaeoisariopsis griseola	2	Kidney bean (*Phaseolus vulgaris*)	Angular leaf spot	–
Phytophthora infestans	5	Tomato (*Lycopersicum esculentum*)	Late blight	–
Rhizoctonia solani	6	Rice (*O. sativa*)	Sheath blight	–
Ustilaginoidea virens	6	Rice (*O. sativa*)	False smut	–

a: the numbers of strains of each species; b: GUS activity positive (+), or negative (–) tested with the blue development assay; all strains of each "–" specie are GUS^-; only one strain of each "+" specie is GUS^+, three GUS^+ strains listed were Cds0701(*Colletotrichum* sp.), Cur0503 (*Curvularia* sp.) and P0704 (*Penicillium* sp.); c, d: unconfirmed *Colletotrichum* genus; two *C.* sp. strains are different, and two strains of each *C.* sp. are uniform in morphology; e: a *Penicillium* sp. strain from air, as a positive control.

Then, we introduced the *gusA* gene into 3 of GUS$^-$ strains, *M. oryzae* strain Guy11, *F. oxysporum* strain Fo0706 and *C. lagenarium* strain Cl0601, and checked the GUS activities of the transformants. A binary vector p1301XGGH containing *gusA* under control of PgpdA and *HPH* cassette was constructed and introduced into the 3 strains respectively. Transformants were collected from hygromycin B containing medium and tested by the GUS assay (Fig. 2). Driven by the fungal promoter PgpdA, *gusA* was expressed strongly. The bright blue transformants and unchanged grey-white wild-type strains were easy to differentiate in each species. Therefore, *gusA* could be used as a marker to identify ectopic transformants in fungi.

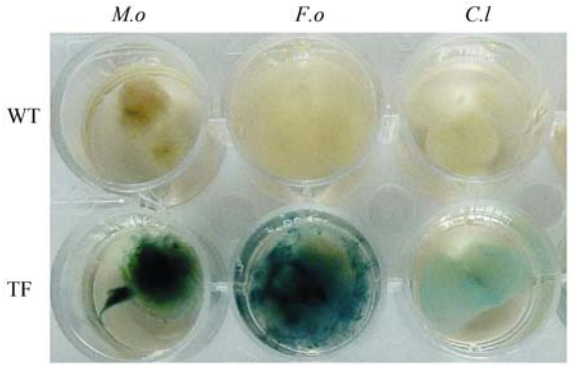
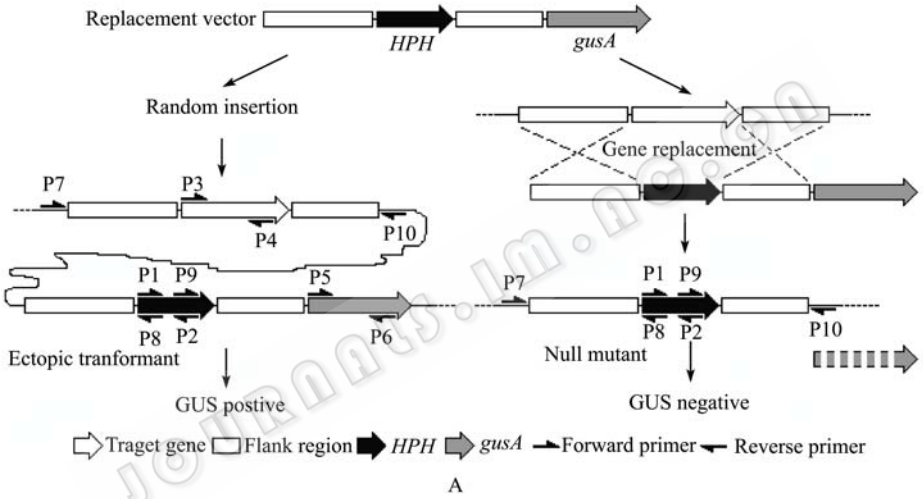


图 2 *M. oryzae*、*F. oxysporum* 和 *C. lagenarium* 的野生型菌株与 *gusA* 转化子菌株的 GUS 活性

Fig. 2 GUS activity in wild-type (WT) and *gusA* transformants (TF) of *M. oryzae*, *F. oxysporum* and *C. lagenarium*. Clumps of WT strains and *gusA* TF of *M. oryzae* (*M. o*), *F. oxysporum* (*F. o*) and *C. lagenarium* (*C. l*) were cultured in liquid medium and subsequently incubated at 37°C in darkness in medium containing X-Gluc·Na.

2.2 GUS-mediated dual screen

The hypothesis of GUS-DS was shown in Fig. 3A.



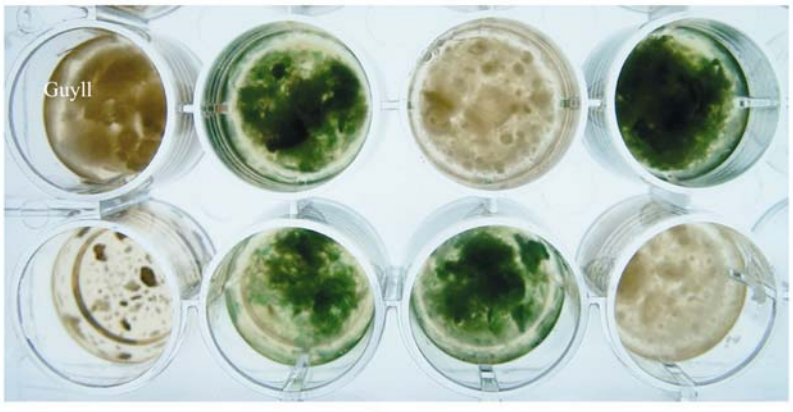


图 3 突变体的 GUS-DS 筛选

Fig. 3 Selection of mutants via GUS-DS. (A) A diagram of GUS-DS. T-DNA carrying a mutant allele (containing 2 flanking regions of target gene and a positive selection marker, such as *HPH*) and the *gusA* cassette was constructed and introduced into fungal nuclei by ATMT. If the construct was integrated randomly into the fungal genome via non-homologous recombination, both *HPH* and *gusA* would remain and would be expressed. If the mutant allele replaced the target gene via homologous recombination, *gusA* would be lost. Therefore, mutants could be selected by hygromycin B and then X- Gluc·Na agents. (B) GUS activity test of Guy11 and transformants. Mycelia were incubated in CM with X-Gluc·Na at 37ºC in darkness for 24 h.

The *gusA* cassette was inserted into the flank of the mutant allele that contained a first selection maker (*HPH* in this work). After transformation, ectopic transformants expressed both *gusA* and *HPH*, while mutants resulting from homologous recombination lacked *gusA*. The ectopic transformants could thus be identified and counter-selected by the GUS assay.

The orthologues of *PEX5* and *PEX7* (assigned as *MGPEX5* and *MGPEX7* respectively in this paper) in *M. oryzae* were used to evaluate the efficiency of GUS-DS. Replacement vectors of *MGPEX5* and *MGPEX7* adapted to GUS-DS was introduced respectively into *M. oryzae* cells by ATMT. All hygromycin B-resistant transformant strains were collected and tested by the GUS assay (Fig. 3B); the GUS^- ones in which were selected as potential mutants and the GUS^+ ones (ectopic insertion) were abandoned.

2.3 M-PCR for confirmation of mutants

For its ease of handling, PCR was used as a primary method for checking mutants. A primer pair was designed inside the region to be replaced (p3 and p4 in Fig. 3A). This primer pair can amplify expected bands in wild-type and ectopic transformants, but not in mutants. However, non-specific amplification products resulted occasionally from unknown reasons. So, mutants confirmed by PCR with only one primer pair often covered false positive cases. Coupled with the GUS-DS, we developed an M-PCR method for confirmation of mutants.

Five primer pairs were designed to amplify *HPH* (p1/p2), the targeted gene (p3/p4), *gusA* (p5/p6), and the recombined 5′ (p7/p8) and 3′ flank regions (p9/p10) (Fig. 3A). To amplify the 5′ recombined flanks, the forward primer p7 was located at the 5′ upward of 5′ flank, and the reverse primer p8 was located at the 5′ end of *HPH*; and to amplify the 3′ recombined flanks, the forward primer p9 was located 5′ end of *HPH*, and the reverse primer p10 was located at the 3′ downward of 3′ flank region. All 5 primer pairs were amplified from genomic DNA of the potential Δ*mgpex5* and Δ*mgpex7* mutants selected via GUS-DS. The primers and the amplificon profiles for Δ*mgpex5* confirmation are shown in Table 3 and Fig. 4 respectively. As expected, only the true Δ*mgpex5* mutants showed the profile of *HPH* (+), *MGPEX5* (–), *gusA* (–), the 5′ recombined flank (+) and the 3′ recombined flank (+).

2.4 Southern blotting analysis

To assess the degree of enrichment of mutants obtained via the GUS-DS method and the reliability of confirmation of mutants by M-PCR, Southern blotting analysis was done by standard procedure. Genomic DNAs were extracted from wild-type, ectopic transformants and potential Δ*mgpex5* (or Δ*mgpex7*) mutants, and hybridized with the probe indicated in Fig. 5 (A, probe 1; C, probe 2). Strains producing hybridizing fragments of a particular size were regarded as the true mutants (Figs. 5B and 5D).

表 3 Δ*mgpex5* 的 M-PCR 验证所用引物

Table 3 M-PCR primers for Δ*mgpex5* confirmation

Name	Sequences(5′–3′)
p1	TGGAGGTCAACACATCAATGCTATT
p2	CTACTCTATTCCTTTGCCCTCGGAC
p3	CTCGGATCAGCTCAGGCACAGA
p4	CCAAGGCGGCCAAAAGATGA
p5	ATGTTACGTCCTGTAGAAAC
p6	AAAGAAGGATTACCTCTA
p7	GGGTTGAGCAAAGGGCAAGTTCC
p8	ATCTGACCAGTTGCCTAAATGAAC
p9	TGTAGAAGTACTCGCCGATAGTGG
p10	GGGTCCACACCAATGTTGCGACC

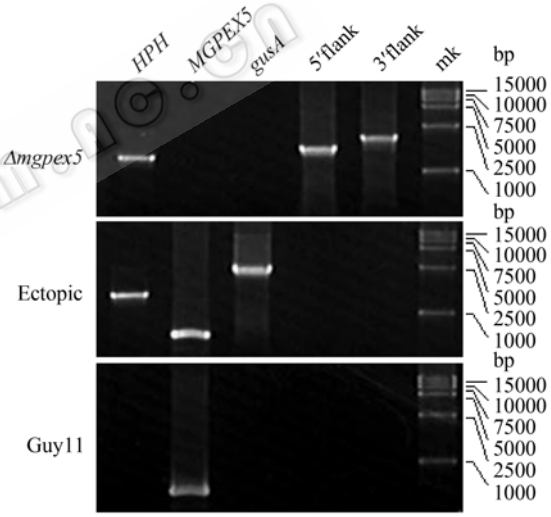


图 4 Δ*mgpex5* 突变体、异位插入转化子及野生型 Guy11 的 M-PCR 检测

Fig. 4 M-PCR test in Δ*mgpex5* mutants, ectopic transformant and wild type Guy11. Five primer pairs (p1/p2, p3/p4, p5/p6, p7/p8, and p9/p10 in Fig. 3A) were used to amplify fragments of *HPH*, *MGPEX5*, *gusA*, 5′ and the 3′ recombined flank region of *MGPEX5* in Δ*mgpex5*, ectopic transformant and Guy11. A DNA marker (mk) indicates the sizes of the fragments.

2.5 Efficiency of GUS-DS and M-PCR

The efficiency and the frequency of false positives of *MGPEX5* and *MGPEX7* obtained by the GUS-DS method were calculated and are given in Table 4. GUS-DS improved the replacement frequency of *MGPEX5* and *MGPEX7* at different levels. The mutants and the number of mutants confirmed by M-PCR

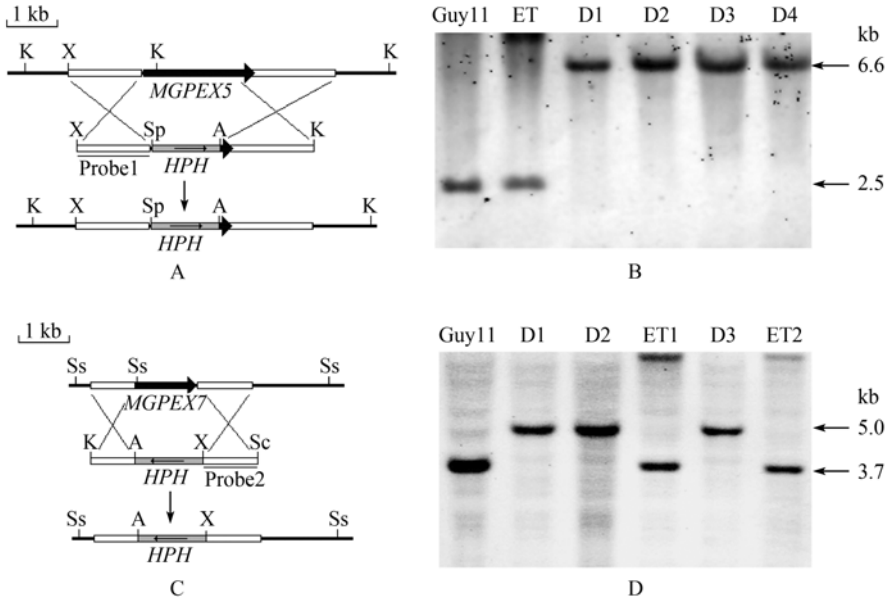


图 5　*MGPEX5* 和 *MGPEX7* 基因替换

Fig. 5　Gene replacement of *MGPEX5* and *MGPEX7*. (A) Homologous recombination between the *MGPEX5* locus and the exogenous mutant allele containing 5′ and 3′ flanking regions of *MGPEX5* and the *HPH* cassette resulted in gene replacement in the mutant genome. Gene replacement led to a loss of a *Kpn* I site and, thus, a 6.6 kb *Kpn* I fragment when hybridized with probe 1; whereas the wild-type allele generated a 2.5 kb *Kpn* I fragment when hybridized with the same probe. (B) Southern blotting analysis of *MGPEX5* replacement. Genomic DNAs extracted from Δ*mgpex5* mutants (D1–D4), the ectopic transformant (ET) and Guy11 were digested with *Kpn* I and hybridized with the 1.4 kb *Xba* I/*Spe* I probe (probe 1). A 6.6 kb hybridizing band appeared in mutants lanes, indicating the homologues recombination events; a 2.5 kb band indicating the wild-type locus was observed in Guy11 lane; and in the ectopic transformant lane, an additional hybridizing band of random size appeared accompanying with the 2.5 kb band, indicating a random insertion of exogenous DNA. (C) Gene replacement of *MGPEX7*. An exogenous mutant allele replaced *MGPEX7*, and *MGPEX7* replacement led to the loss of an *Ssp* I site and, thus, a 5.0 kb *Ssp* I fragment when hybridized with probe 2; and the wild-type allele generated a 3.7 kb *Ssp* I hybridizing fragment. (D) Southern blotting analysis of *MGPEX7* replacement. Genomic DNAs from Δ*mgpex7* mutants (D1–D3), ectopic transformants (ET1 and ET2) and Guy11 were digested by *Ssp* I and hybridized with the 1.0 kb *Xba* I/*Sac* I probe (prob2). A 5.0 kb hybridizing band indicating *MGPEX7* replacement appeared in the mutants lanes; a 3.7 kb band indicating the wild-type locus appeared in the Guy11 lane; and a random-sized band together with a 3.7 kb one indicating the ectopic insertion was observed in the ectopic transformants lanes. Restriction sites: A: *Apa* I; K: *Kpn* I; Sc: *Sac* I; Sp: *Spe* I; Ss: *Ssp* I; X: *Xba* I.

表 4　*MGPEX5* 与 *MGPEX7* 基因的 GUS-DS 及 M-PCR 的筛选效率

Table 4　Efficiency of GUS-DS and M-PCR for *MGPEX5* and *MGPEX7*

Target gene	HR[a]	GUS^{-}[b]	Mmp[c]	Ms[d]	FP[e]	fGR[f]	f′GR[g]	fFP[h]	Flank region[i]
MGPEX5	73	53	48	48	5	65.8%	90.6%	9.4%	1.5 kb; 1.8 kb
MGPEX7	93	35	29	29	6	31.2%	82.8%	17.1%	0.8 kb; 1.0 kb

a: HR, total number of hygromycin B-resistant transformants;
b: GUS^{-}, the number of transformants without GUS activity among HR;
c: Mmp, the number of mutants confirmed by M-PCR method;
d: Ms, the number of mutants confirmed by Southern blotting;
e: FP, the number of false positive transformants (hygromycin B-resistant, without GUS activity, but out of Ms);
f: fGR, the frequency of gene replacement without selection by GUS staining, percentage of Ms (or Mmp) among HR;
g: f′GR, the improved frequency of gene replacement by GUS-DS, percentage of Ms (or Mmp) among GUS^{-};
h: fFP, the false positive frequency of GUS-DS, percentage of FP among GUS^{-};
i: Flank region, the sizes of flank regions in replacement vectors.

(Mmp) were the same as those confirmed by Southern blotting (Ms), so these two methods are equally reliable. The gene replacement frequency of *MGPEX5* is higher than that of *MGPEX7*.

In view of the crucial roles of *PEX5* and *PEX7* in peroxisome biogenesis, the probable involvement of *MGPEX5* and *MGPEX7* in lipid metabolism in *M. oryzae* was checked by a lipid utilization test. The clumps of Δ*mgpex5* and Δ*mgpex7* mutants, ectopic transformants and wild-type Guy11 were incubated on

medium with lipids as sole carbon source (FAM). After 4~5 days, Guy11 and the ectopic transformants formed extensible colonies; while only a few hyphae grew from the inoculated clumps of Δ*mgpex5* and Δ*mgpex7* mutants, which did not produce colonies (Fig. 6). The strains capable or incapable of growth on FAM were completely consistent with the ectopic transformants or mutants confirmed by Southern blotting or M-PCR. The lipid utilization test gave a phenotypic confirmation of gene replacement events in the Δ*mgpex5* and Δ*mgpex7* strains, and indicated that *MGPEX5* and *MGPEX7* were functional homologues of *PEX5* and *PEX7*.

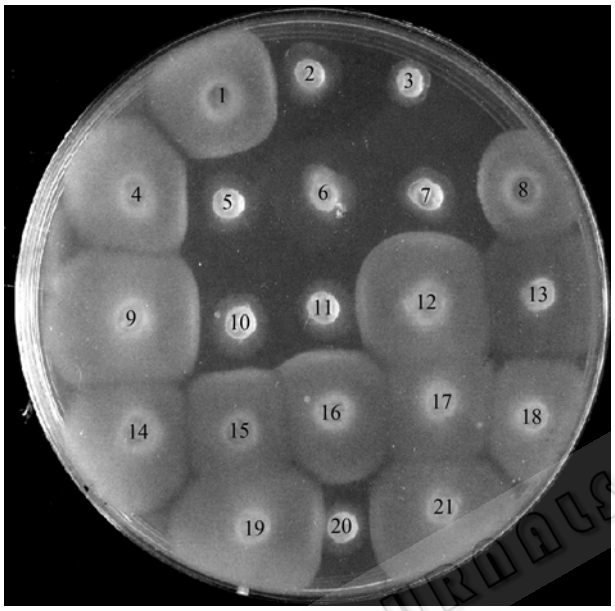


图 6 Δ*mgpex5* 与 Δ*mgpex7* 突变体、异位插入转化子和 Guy11 在 FAM 培养基上的生长情况

Fig. 6 Growth of Δ*mgpex5* and Δ*mgpex7* mutants, ectopic transformants and Guy11 on FAM. In medium with lipid as the sole carbon source (FAM), Guy11 (21), ectopic transformants of *MGPEX5* (1, 4, 8) and *MGPEX7* (9, 12–19) grew normally, while Δ*mgpex5* (2, 3, 5–7) and Δ*mgpex7* mutants (10, 11, 20) did not.

3 Discussion

Although several DS systems have been already applied in fungi, the GUS gene was first used as a negative selection marker. In most DS systems, the negative selection was achieved via genes which code some toxic products to suppress the ectopic transformants. But different fungal species or strains often have different tolerance to a same toxic compound. The *gusA* under the control of a universal fungal promoter is able to produce the blue reaction in various fungal strains by similar procedures. Screen via GUS is thus easier to achieve with a uniform system in different fungi. The selection pressure of some toxic reagents tends to increase false positive rates. GUS selection avoids this problem to some extent, for the GUS substrate is harmless to fungal cells. GUS-DS is unable to completely avoid the interference of false positives. Confirmation by PCR or Southern blotting for mutants is still indispensable; fortunately, our M-PCR method makes it simple and rapid.

The complete lack or extreme lowness of endogenous GUS activity in many fungal species make it possible to apply the GUS-DS in more cases. *P. canescens* was reported as a GUS^+ genus and *gus* genes have been identified in *P. canescens*, *G. zeae* and an *Aspergillus* sp. [28, 29]. In our study, GUS activity was detected in a *Penicillium* sp. strain, but not in *G. zeae* (Table 2), which may be because the blue development method was not sensitive enough to detect some low levels of GUS activity. Despite this, *gusA*-mediated screen may be applicable to the fungal species in which the exogenous GUS activity from transgenic *gusA* and the endogenous can be differentiated easily by the development of blue color.

GUS staining for fungi is not convenient on agar plate [16, 30]. We solved this problem by processing it in liquid, but brought a more transfer step from agar to liquid. So, our next goal is to achieve the GUS-DS directly on agar plate by using stronger promoter or inserting secretive signal before *gusA* ORF. And this work is still ongoing.

Mutants could be enriched about 2000-fold in mammalian cells by a positive/negative method [31]. In practice, however, the enrichment effect was often only 10-fold to 100-fold [32]. In this work, the enrichment for homologous recombinants by GUS-DS in *M. oryzae* was only 1.4-fold (from 65.8% to 90.6%) to *MGPEX5* and 2.7-fold (from 31.2% to 82.8%) to *MGPEX7* (Table 3). This low degree of enrichment resulted, in part, from the high frequency of homologous recombination (65.8% and 31.2%) of the 2 genes, but this method is still useful for reducing the workload in the screen of mutants. *Agrobacterium*-mediated transformation was reported to be more suitable for gene replacement and often produces a higher recombination frequency than other transformation techniques in a variety of filamentous fungi [33–35]. However, 65.8% of *MGPEX5* is quite a high value. In filamentous fungi, the length of a homologous sequence affects the frequency of homologous recombination, which is likely to be the reason why the replacement of *MGPEX5* produces such a high frequency, and why the replacement

frequency of *MGPEX5* is higher than that of *MGPEX7*. In our studies of gene replacement by ATMT, the frequency of homologous recombination ranged from 3% to 60%. It is predictable that GUS-DS can contribute more to the enrichment of mutants with low-frequency recombination. So, GUS-DS would be more useful for some low-yield expression genes, some fungal species with low-frequency recombination, or some transformation methods producing low-frequency recombination.

The availability of genome sequences for a number of filamentous fungi, including *M. oryzae*, *G. zeae* and *A. nidulans*, opens a new research avenue, and a number of genes with unknown function should be investigated. Simple, fast and efficient procedures for the screen of mutants will be important for the application of targeted gene replacement in more fungal species. We have already generated targeted mutants of more than 30 genes by using GUS-DS and M-PCR confirmation in *M. oryzae*, showing that these procedures are useful. We hope these procedures will drive the application of TGR in fungal genomics.

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