International Scholars Journals

African Journal of Botany ISSN: 3519-3824 Vol. 8 (2), pp. 001-008, February, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Evidence of resistance gene markers parasexual recombination in *Magnaporthe grisea*

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Accepted 16 October, 2019

A selectable marker gene conferring resistance to bialaphos (*Bar*) was introduced into rice blast isolate 95-8-36 and another conferring resistance to hygromycin (*Hph*) into isolate P32-1 of *Magnaporthe grisea* by *Agrobacterium tumefaciens*- mediated transformation (ATMT) to demonstrate exchange of DNA. Many of the transformants of 95-8-36 and P32-1 were mixed by grinding and cultured on oat agar media. Spores were collected with sterile water and inoculated in PSA selection medium with hygromycin and glufosinate in order to screen the recombinants. Some isolates were obtained from the medium and confirmed to have *Bar* and *Hph* genes by PCR technique, indicating that *M. grisea* made parasexual recombination under the artificial conditions. The recombinants could inherit the double resistance stably such that their growth rate and sporulation ability also resemble their original isolates.

Key words: Parasexual recombination, *Magnaporthe grisea,* resistant gene mark, *Agrobacterium tumefaciens*-mediated transformation (ATMT).

INTRODUCTION

Magnaporthe grisea (Hebert) Barr [anamorph *Pyricularia grisea* (Cooke) Sacc] causes rice blast disease, which is the most destructive disease of rice production around the world and one of the most important rice diseases in northern and southern China.

At present, the effective way to control this disease is through the application of resistant varieties. However, the pathogenicity of *M. grisea* has a very high variability under natural conditions because of its complex population structure and heredity, which resulted in the breakdown of their blast resistance within three to five years after these resistant varieties were introduced into fields (Sweigard et al., 1995). Therefore, the investigation of its variation mechanisms is becoming a focal research point in the world.

Regarding the variation in the pathogenicity of *M. grisea*, it is very possible that it is caused by mutation, chromosome number changes, and asexual recombination that happened during the parasexuality. It is difficult to use the classical genetic methods to uncover the hereditary property of *M. grisea* (Crawford et al., 1986; Leung and Tega, 1988). Exploring whether *M. grisea* has parasexual recombination and mutate from this recombination is one of the ways to understand its variation.

Parasexuality is the major cause of *M. grisea* variation because it has no sexual stage, or because the sexual stage uncommonly occurs under field conditions. Although the parasexual recombination in some fungi has been investigated clearly (Paccola-meirelles and Azevedo, 1991; Souza-paccola et al., 2003), many researchers still

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view this way of reproduction as one meant for *M*. *grisea* (Namai and Yamanaka, 1982; Yamasaki and Niizeki, 1965).

Up till date, the asexual reproduction phenomenon of *M*. *grisea* has been well documented (Genovesi and Magill, 1975; Fatemi and Nelson, 1978; Crawford et al., 1986; Chuanqing and Mingquo, 2005; Zhengguang and Xiaobo, 2000; Yanli et al., 2003), while only few reports (Zeigler et al., 1997, 1998; Noguchi et al., 2006) are available for parasexual recombination of this fungus. So studying the parasexual recombination of *M*. *grisea* is very important to discover the variation mechanism of rice blast.

In this study, we aimed to demonstrate the parasexual recombination of *M. grisea* by using the resistant gene markers. Through screening the resistance genes of recombinants to reveal that *M. grisea* strain can occur through parasexual recombination under artificial conditions. Using resistance gene markers as evidence to study the parasexual recombination of *M. grisea* may be a good way to research the pathogenicity of *M. grisea.*

MATERIALS AND METHODS

M. grisea isolates

M. *grisea* strain 95-8-36 [*Mat1-2* (a)] and P32-1 [*Mat1-1* (A)], two field isolates which have opposite pathogenic patterns, collected from Yunnan province in China in 1995, were used as wild type isolate and were cultured on complete medium (CM) that were stored in our laboratory.

Vector strains

Escherichia coli strain DH5α and *Agrobacterium tumefaciens* EHA105 were stored by our laboratory and *A. tumefaciens* C58C1 presented by Yasuyuki Kubo at the Kyoto Prefectural University (Kyoto, Japan), were used as a host for plasmid amplification. Plasmid pBIG4MRBrev with *Bar* gene and plasmid pBIG2RHPH2 with *Hph* gene, which are all binary vectors containing *NPTⅢ* gene (Figures 1 and 2), were presented by Yasuyuki Kubo.

Transformed resistant marker gene

Plasmid pBIG4MRBrev, which is a phosphinothricin resistance vector containing *Bar* gene, transformed into *M*. grisea strain 95-8-36 and produced *Bar*-resistant transformant 95-8-36B by using *A. tumefaciens* EHA105 harboring a plasmid pBIG4MRBrev. In another way, transformed *A. tumefaciens* C58C1 harboring a plasmid pBIG2RHPH2, which is a hygromycin resistance vector containing *Hph* gene, transformed into *M. grisea* strain P32-1 and produced *Hph*-resistance transformants P32-1H. The whole process of ATMT followed the method of Yuan Yuan et al. (2007); Hongyu et al. (2003).

Detection of the resistant gene in transformants

Before the two kinds of transformant strains recombined, the presence of *Bar* gene in transformants 95-8-36B and *Hph* gene in transformants P32-1H were confirmed by PCR. Based on the 1.4 kb *Hph* fragment in the plasmid pBIG2RHPH2 and 500 bp *Bar* fragment in the plasmid pBIG4MRBrev, we designed the *Hph*-specific primers: *Hph*1(5′-ACGTTAACTGATATTGAAGGAGCAT-3′) and *Hph*2(5′-ACGTTAACTGGTTCCCGGTC-3′) and *Bar*-specific primers: *Bar*1(5′- AAGCACGGTCAACTTCCGTA-3′) and *Bar*2(5′-GAAGTCCAGCTGCCAGAAAC-3′) in order to screen the resistant gene in these transformants.

Recombination

In order to promote recombination rate among two kinds of different transformant strains, about 400 transformants from 95-8-36B and 400 transformants from P32-1H were mixed to conculture in PSA medium at 28°C in the dark for five days under sterile condition, ground in the PSA mediums that had grown the mixed transformant strains and spread on oat agar media (OAM).

After the aerial mycelia grew into full petri dish, they were scraped away and the plate was kept under continuous light for sporulation for two days. Spores were collected with sterile water and 300 µL liquid spores were inoculated in PSA selection medium with 200 µg/mL hygromycin and 20 µg/mL glufosinate in order to screen the recombinants.

Examined resistant gene marker of recombinants

Extracted the genomic DNA of a part of recombinants using the method described by (Yueqiu, 2000) DNA samples were amplified with *Bar* gene-specific primers and *Hph*-specific primers, respectively.

Stability of recombinants

To inspect the stability of the recombinant strains, 50 randomly selected recombinant strains were successively cultured on PSA medium without hygromycin B and glufosinate from the second to the ninth generation.

The first generation and the 10th generation were cultured on selection medium (with 200 µg/mL hygromycin and 2 0µg/mL glufosinate), respectively, to test resistance.

Growth rate and colony morphology of recombinants

To inspect radial colony growth rate of recombinants, taking wild type strains 95-8-36 and P32-1, transformant 95-8-36B and P32-1H as control, randomly selected recombinant strains were cultured on PSA medium at 28°C, measured average thalli diameters daily for up to 10 days with 4 replicates. Then, observed their colony morphology.

Sporulation ability assays

Using wild type strains 95-8-36 and P32-1 as control, taking 95-8-36B, P32-1H and recombinant strains were cultured on OAM in the dark at 28°C.

The aerial mycelia were scraped away once they spread onto the full petri dish and the plates were kept under continuous light at 28°C to induce sporulation for two days. Spores were collected with sterile water and counted with a haemacytometer under a microscope.

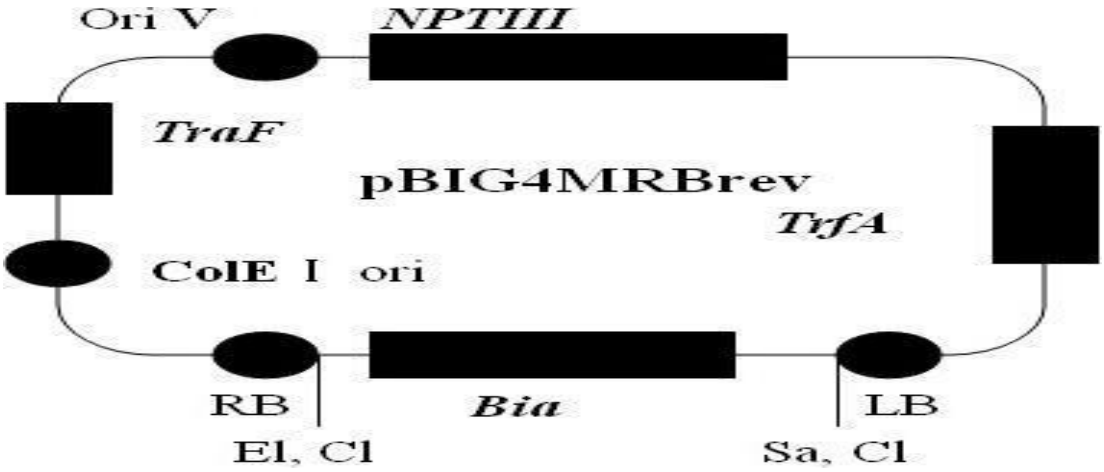

Figure 1. Map of plasmid pBIG4MRBrev.

Figure 2. Map of plasmid pBIG2RHPH2

RESULTS

M. grisea transformation

In this study, plasmids pBIG4MRBrev with *Bar* gene and pBIG2RHPH2 with *Hph* gene were used to transform wild type *M. grisea* strains: 95-8-36 and P32-1, mediated by *A. tumefaciens* EHA105 and *A. tumefaciens* C58C1, respectively. We obtained two kinds of transformant strains, 95-8-36B with *Bar* resistance gene mark and P32-1H with *Hph* resistance gene mark (Figures 3 and 4). The transformation efficiency was 500 to 1000 transformants per 1×10^6 conidia.

Recombination

Transformants 95-8-36B and P32-1H were mixed to conculture on PSA (Figure 5), their hyphae inosculated on the medium. The recombinants which contained two antibiotic-resistance genes were obtained by screenings with selection medium [selection medium (SM), PSA with 200 µg/mL hygromycin and 20 µg/mL glufosinate]. The recombinant could grow but the wild type isolate, transformant 95-8-36B and transformant P32-1H were suppressed on the SM (Figure 6).

PCR examination

The DNA samples of the recombinants, transformants 95-8-36B and P32-1H were amplified with *Bar*-gene (450 bp) and *Hph*-gene (1500 bp) specific primers, respectively. All of the ten recombinants showed 450 and 1500 bp band corresponding to the *Bar*-gene and *Hph*-gene, respectively. However, transformants 95-8-36B only

Figure 3. Transformants of 95-8-36 on CM with 15μg/mL of glufosinate.

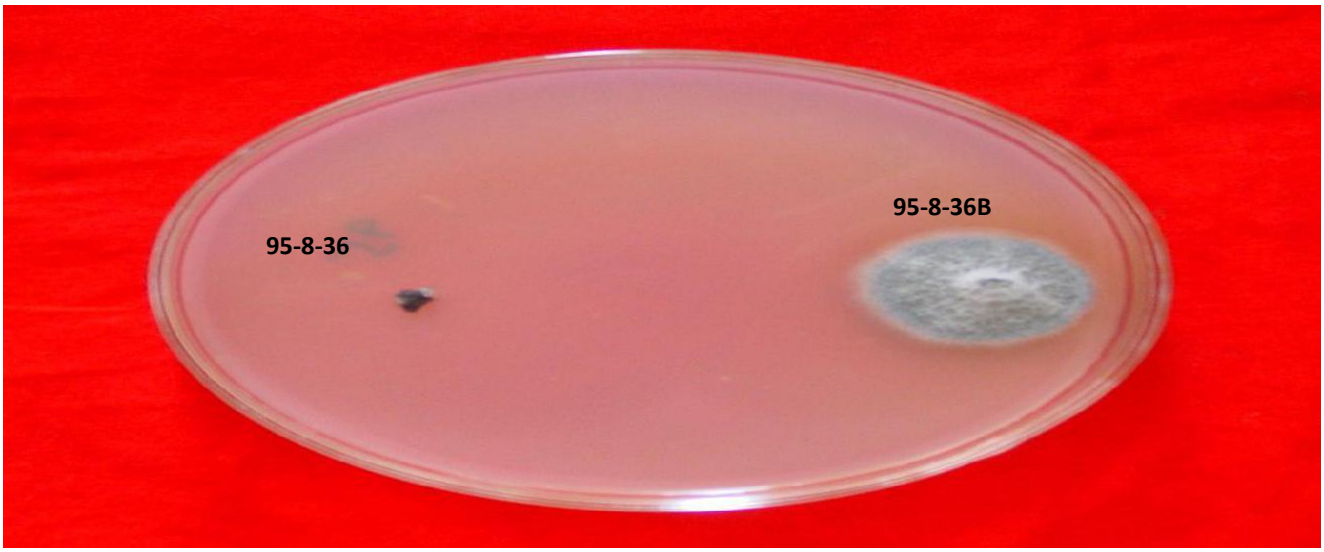

Figure 4. 95-8-36 and its transformant on PSA with 20μg/mL of glufosinate (after 3 days).

contained a 450 bp band and transformants P32-1H contained a 1500 bp band (Figures 7 and 8).

Stability of recombinants

To inspect the stability of the recombinants, 50 recombinants were randomly selected to successive culture in PSA medium. The first generation and the 10th generation were cultured on SM with 200 μg/mL hygromycin and 20 μg/mL glufosinate, respectively. The 10th generation could still grow on SM, so the recombinant was stable for the ten generations.

Biomorphic examination of recombinant strains

To examine the biomorphic features of recombinants, using WT95, WTP32, 95B and P32H as control, contrast results in the growth rate sporulation ability and strain morphology of the recombinants are shown in Table 1 and Figure 9. From the thalli diameters measured daily for up to 10 days with 4 replicates (detail dates were not show), found recombinants growth rate showed no significant differences (at $P = 0.05$) from the wild type isolate 95-8-36 and P32-1, transformants 95-8-36B and P32-1H (Table 1). Contrasting the sporulation of the recombinants with the wild type isolate 95-8-36 and P32-1, transformants

Figure 5. Two kinds of transformants with *Bar* and *Hph* genes, respectively, were cocultuerd on PSA medium.

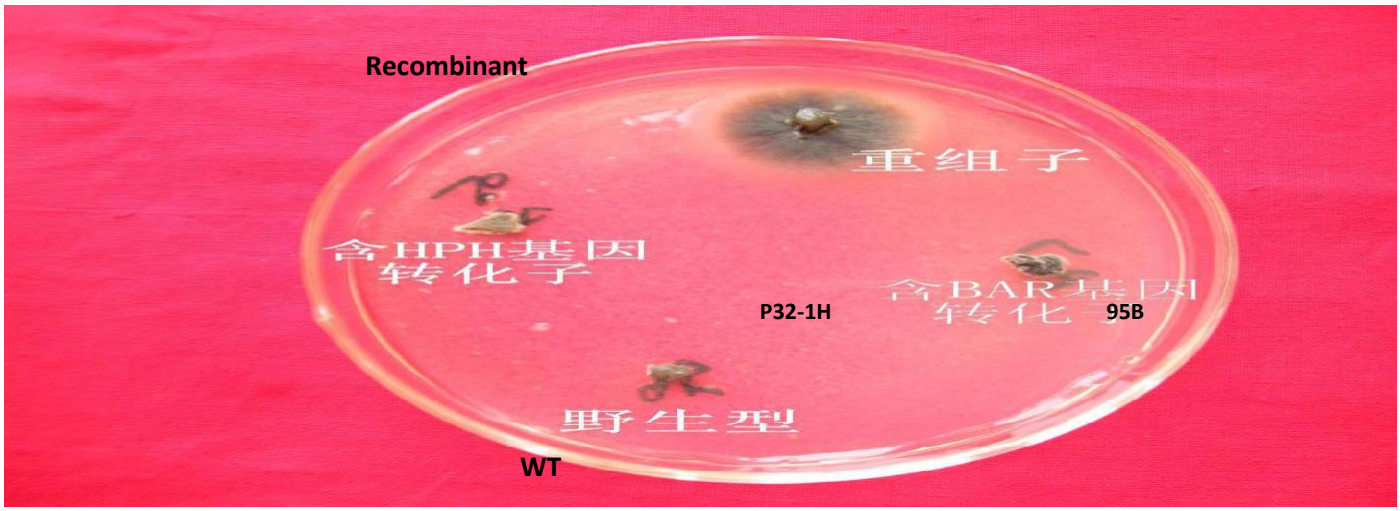

Figure 6. The wild type isolate, 95-8-36B-1, P32-1H-1 and recombinant on SM.

95-8-36B and P32-1H, the recombinants ($1.65\pm0.76\times10^6$ ／ mL) were less than the wild type isolate P32-1($3.29\pm1.43\times10^6$ ／ mL) and the transformants 95-8-36B($3.92\pm3.04\times10^6$ ／ mL), but there was no significant difference between the wild type isolate 95-8-36 and transformant P32-1H at P = 0.05 (Table 1). A comparison of the recombinants morphology with those of the wild type and transformant strains showed that the morphology of the recombinants was more similar to the wild type isolate P32-1 and transformant P32-1H (Figure 9).

DISCUSSION

Parasexuality was counted as the primary reason leading to genetic variation of the imperfect fungi with an uncommon perfect stage in nature. However, it was still a hypothesis that parasexual recombination causes pathogenic variants of M. grisea because of lack of direct

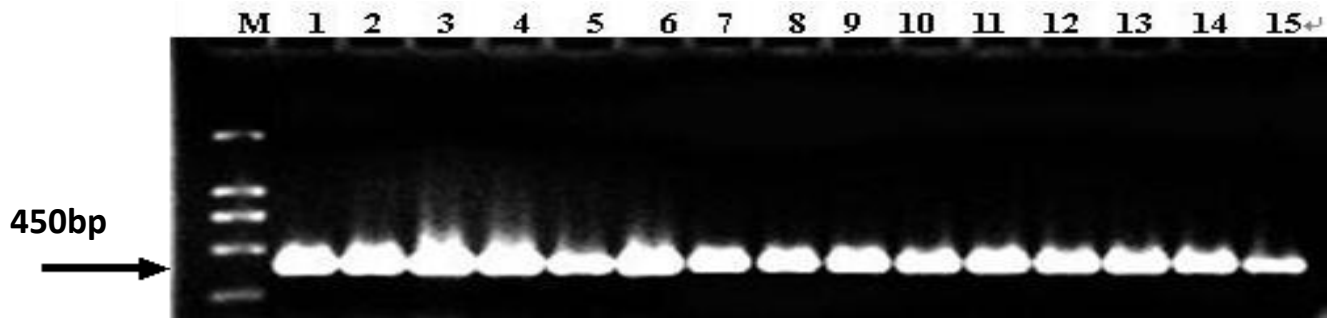

Figure 7. PCR products of mutants amplified with *Bar* gene primers (LaneM is DR2000 Mark, Lanes 1 to 4 are transformants 95-8-36B, the rest are recombinants).

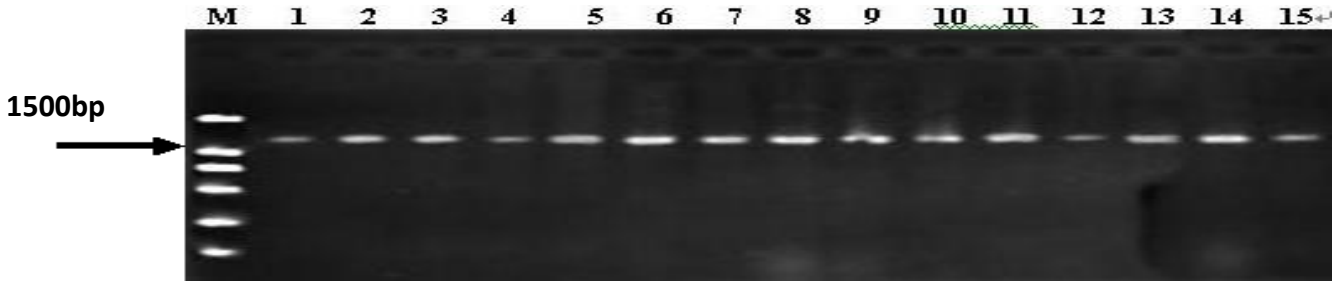

Figure 8. PCR products of amplified with *Hph* gene specific primers. (LaneM is1kb Mark, Lanes 1 to 4 are transformants P32-1H the rest are reconbinants).

Table 1. Comparison of the growth speed between the wild type and transformant strains.

Strain	Thalli diameter[Y] (cm) ($\overline{X}$ ±SD)			Sporulation[Z]
	4th day	7th day	10th day	(×10^6 / Ml)
Wild type 95-8-36	2.83±0.06 ab	5.23±0.12 a	7.47±0.15 a	1.56±0.79 c
Wild type P32-1	2.80±0.10 ab	5.43±0.12 a	7.60±0.10 a	3.29±1.43 ab
T.95-8-36B	2.87±0.06 a	5.30±0.17 a	7.63±0.21 a	3.92±3.04 a
T.P32-1H	2.60±0.14 b	5.35±0.21 a	7.50±0.28 a	2.48±2.15 bc
Recombinant	2.67±0.21 ab	5.13±0.15 a	7.33±0.06 a	1.65±0.76 c

Y: Assays of radial growth rate was described in detail in the text. Average of four replicates±standard deviation. Different letters in each data column indicate significant differences at *P = 0.05*.
Z: Different letters in each data column indicate significant differences at *P = 0.05*.

evidence, especially in the wild type.

The study of parasexuality in fields was hindered due to the fact that it was banned by laws that direct research parasexuality on plants. In addition, the requirement of research materials restrained this study in the field. For instance, in this study, the use of near-isogenic lines (NILs) and the near-isogenic pyramid lines with genetic uniformity was required, and the tested isolates should have a pathogenicity complementation with tested NILs. Although the isolates that came from hyphal fusion were easily obtained in the laboratory, those isolates had either unstable heredity, or unclear genetic backgrounds, or obtained recombinants were not related with the pathogenicity of *M. grisea* such that it could not be used for the study. Moreover, it required strict experimental conditions, such as removing the possibility of cross contamination between tested isolates and the need to exclude site mutation.

The parasexual recombination of *M. grisea* in artificial culture has been demonstrated with drug markers (Zhengguang and Xiaobo, 1998, 1999), auxotrophic mutants that were induced by physical or chemical

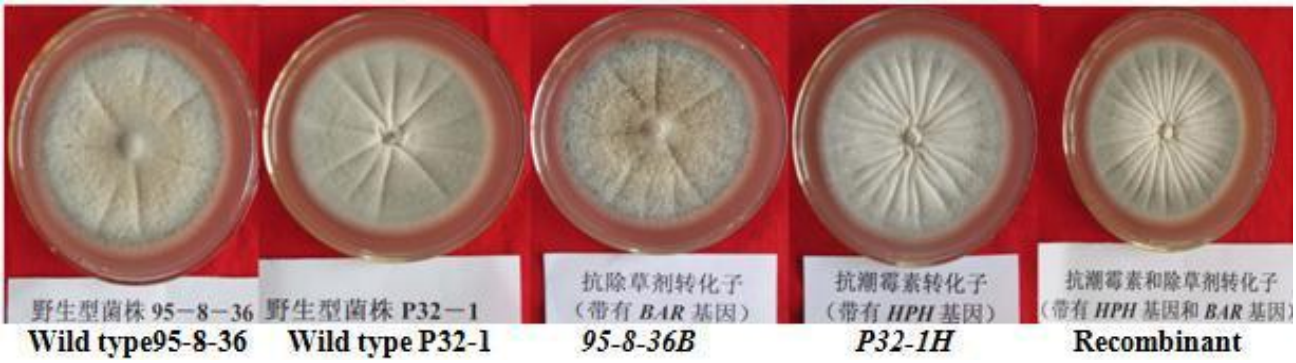

Figure 9. Comparison of the colony morphology between the recombinant and control isolates.

mutagen (Cailin et al., 2002), the auxotrophic mutation complementation test (Crawford et al., 1986; Chuanqing and Mingquo, 2005), and the use of the repetitive elements MGR585 and MAGGY as molecular markers (Zeigler et al., 1997, 1998).

Since the direct evidence of the pathogenic variants of *M. grisea* due to parasexual recombination in the field has not been reported, it was difficult to detect if parasexual recombination has occurred using auxotrophic mutation or drug as markers. Furthermore, because of the high level of variation in *M. grisea*, these two kinds of technology are often difficult during the experiment. In this study, to obtain double resistant recombinants, *Bar* and *Hph* resistance gene markers were directly inserted into two *M. grisea* strains which have opposite pathogenic patterns, respectively, by ATMT.

The result showed that the recombinant strains had obtained *Hph* and *Bar* resistance, and the parasexual recombination could take place on the culture medium. It also revealed that in the field, parasexual recombination of *M. grisea* was possible. This method of study was not only guaranteed by the stability of genetic transformants, but was also easily operated and had high transforming efficiency. Even more importantly, gene markers of obtained recombinants could inherit stability, the genetic background of recombinants was clear so that it was greatly convenient to screen and test the recombinant strains and supply ideal isolated parasexual recombination for study in future.

In addition, the experimental method of obtained recombinants was simple and easy to do, and also improved the efficiency of recombination. This technological system has great significance in guiding other researchers to do similar studies in future. Although it has been reported that *M. grisea* can be parasexually recombined, the researches about the biological characteristics of the recombinants are rare (ChuanQin and Mingguo, 2006).

Some biological characteristics of recombinant strains were carried out in this study. Recombinants could grow well in a medium, and there were no differences between parent isolates in colony morphology; growth rate and conidia sporulation capacity of recombinants were similar with those of parent isolates. It was revealed that the recombinants could survive in the field and their genetic structures were stable. It was a good way of researching into *M. grisea* pathogenicity.

Parasexual recombination was important for the pathogenicity variation and genetic diversity of *M. grisea.* It is possible that different isolates with different pathogenicity simultaneously infect the plants, form a lesion, and then a genetic exchange among these isolates possibly occur.

Further studies are needed to demonstrate how much effect parasexuality has on the genetic diversity of *M. grisea,* how often the rate of recombination appears in the field and how fit the progeny of parasexual recombination is in the field.

ACKNOWLEDGMENTS

This work was supported by a grant from the National High Technology Research and Development Program of China (863 Program) (No.2002AA245041, 2003GP06) and National Science Foundation of China (30260006).

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