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RESEARCH ARTICLE

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Screening and identification of mutants of *Magnaporthe grisea* by REMI

Abstract The plasmid pUCATPH was used to establish a transformation system in wild-type isolate M131 of *Magnaporthe grisea*. Six hundred and thirty-nine transformants were obtained by restriction enzyme-mediated integration (REMI) with *hygromycin* B (hyg B) resistance as a tag. Morphological analysis of two of the REMI mutants confirmed that they produced little melanin under black light and continued for three generations. Pathogenicity identification of six mutants screened proved that they made pathogenicity changes on three sets of differential varieties with different resistance genes. Rep-PCR analyses showed that two morphological mutants and two pathogenicity mutants differed from wild-type isolate M131 at the molecular level. RFLP analyses were performed to study the four mutants at the molecular level and the integration sites of the plasmid DNA. The results showed that the plasmid was inserted into all four mutants and that the insertion sites were random.

Keywords *Magnaporthe grisea*, restriction enzyme mediated integration (REMI), morphological mutant, pathogenicity mutant

1 Introduction

Rice blast, caused by *Magnaporthe grisea* (Hebert) Barr. (anamorph: *Pyricularia grisea* Sacc.), is one of the most important diseases of rice and can cause severe losses in most rice-growing environments (Crawford et al., 1986; Fang and Guo, 1991; Lee et al., 2006; Ou, 1985). It is difficult to cultivate and select rice varieties resistant to *M. grisea* because the fungus has a high aberrance rate in nature (Leung and Tega, 1988). Among isolated pathogens of rice, hundreds of strains have been identified and defined by their virulence or avirulence toward particular rice cultivars. Cultivars of rice that differ from one another by the presence or absence of dominant blast resistance genes have been developed**,** but the resistance of any one of these cultivars is only against certain strains of the pathogen (Crute, 1986). The fungus shows a high degree of variability in nature; new strains frequently appear with the ability to infect previously resistant rice cultivars (Couch et al., 2005). Individual *M. grisea* strains produce distinctive and reproducible symptoms on particular host plants under a given environmental regime (Ou, 1985).

Pathogenicity is a complex phenotype, involving such distinct components as infection efficiency to determine the lesion number, rate of lesion development, extent of colonization determining the lesion size and efficiency of sporulation (Pia et al., 1999). Genetic analysis of host-pathogen interactions, accomplished mainly in systems with obligate fungal pathogens such as the rusts and the mildews, confirms the complexity of the interactions. Quantitative pathogenicity factors determine at least some aspects of the interaction between the pathogen and its host (Crute, 1986). “Major genes”, single genes with large effects on a host-pathogen interaction, are also common. Except for preliminary reports on the genetic analysis of host specificity, little is known about the genetic basis of *M. grisea* pathogenicity and host specificity.

In many cases, the scope of insertional mutagenesis has been extended to include restriction enzyme-mediated integration (REMI) (Shi et al., 1995). Initially, the method was developed for *Saccharomyces cerevisiae* (Manivasakam and Schiestl, 1998), but it has also been used successfully for other fungi (Bolker et al., 1995; Kuspa and Loomis, 1992; Lu, 1994). In our experiment, morphological mutants and pathogenicity mutants were obtained by transformation. All of the transformants obtained using restriction enzymes had major changes in their genomes as shown by PCR and RFLP. Our research may provide a basis for searching for the related gene of pathogenicity change and cloning the avirulence gene of *M. grisea* (Muriel et al., 2002).

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2 Materials and methods

2.1 Strains and plasmids

The transformation experiments were performed with the *M. grisea* wild-type strain M131 provided by Peng Y L (China Agricultural University). The plasmid pUCATPH, which carries the *hygromycin* B (hyg B) resistance gene, was used to transform *M. grisea*.

2.2 Media and culture conditions

For DNA isolation, the fungal strains were grown in 100 mL of CM liquid medium optimized for *M. grisea* for three days at 37°C on a rotary shaker at 200 r/min (Pia et al., 1999).

2.3 DNA isolation

Genomic DNA was isolated according to the method of Cenis (1993). The mycelium was harvested by filtration through sterile filter paper, washed with sterile distilled water, frozen in liquid nitrogen and lyophilized for 24 h. The lyophilized mycelium was then ground to a fine powder. Plasmid DNA was extracted following Cenis (1993).

2.4 DNA transformation

Transformation of *M. grisea* was conducted using a circular or linearized plasmid pUCATPH with or without the addition of restriction endonucleases (Parsons et al., 1987). Protoplasts were obtained as described previously (Zhou et al., 2000). Ten μg of plasmid DNA and 50 μL of polyethylene glycol (PEG) (25%PEG 6 000 in STC) were added to mixtures containing 100 μL of protoplasts at a concentration of 5×10^7/mL in STC [1.3 mol/L sorbitol, 10 mol/L Tris-HCl (pH 7.5), 10 mol/L $CaCl_2$]. For REMI transformation, 10 μg of plasmid DNA was incubated with a restriction endonuclease (*Hin*d III, *Sac* I, *Kpn* I or *Eco*RV) in 50 μL of PEG 6 000 for two hours and then mixed with 50 μL of protoplasts. The transformation mixture was incubated on ice for 25 min, then an additional 2 μL of PEG 6 000 was added. The mixture was mixed and kept at room temperature for 10 min before 4 mL of STC buffer was added.

2.5 Identification of pathogenicity and morphology of transformants

To observe the morphological changes of the transformants, fungi were cultivated on a spore plate for seven days at 26°C under black light. Three sets of differential varieties were used to identify pathogenicity of the transformants (Zhou, 2000).

2.6 Molecular analysis of the transformants

Rep-PCR was carried out according to the method of Kachroo et al. (1994) (George et al., 1998). The number of copies of integrated pUCATPH in *M. grisea* transformants was determined by Southern blot hybridization. Genomic DNA from the transformants and the wild-type M131 were digested with *Hin*d III, *Sal* I and *Kpn* I (Sambrook et al., 1989), cut once and twice respectively, in pUCATPH. Hybridization signals of *Hin*d III-digested DNA were analyzed to determine the copied number of integrated vector. The restricted genomic DNA was transferred to Hybond N^+filters (Amersham, Braunschweig, Germany). Filters were hybridized and probes were labeled with DIG DNA labeling and detection kit (Roche, No. 1093675) according to the manufacturer's protocol.

3 Results

3.1 Transformation frequency

Protoplasts of the wild-type *M. grisea* M131 were transformed conventionally by a circular or linearized plasmid DNA of the vector pUCATPH and by a modified REMI procedure in which 40, 80, 200, 400 and 600 U/mL of one of the restriction enzymes (*Hin*d III, *Sal* I and *Kpn* I), which linearized the vector, were added to each transformation mixture (Table 1). When using circular plasmid DNA without restriction enzymes, the transformation rate was very low. The addition of *Hin*d III and *Sal* I to the circular plasmid, especially the addition of *Hin*d III at 200 U/mL, significantly increased the transformation efficiency. Reproducibly high numbers of transformants were also obtained with *Sal* I-linearized pUCATPH, with or without heat-inactivation of the restriction enzyme. Larger amounts of restriction enzyme (600 U/mL) reduced the transformation frequency. *Kpn* I had no significant effect on transformation frequency using linearized plasmid.

3.2 Screening for REMI mutants

Approximately 639 transformants obtained by REMI were screened. Among the REMI transformants, two strains

Table 1 Transformation frequency of circular plasmid and linearized plasmid

Enzyme	Number of transformants						
	Circular plasmid/μg	Linearized plasmid/μg	Amount of enzyme in linearized plasmid/U·mL^{-1}				
			40	80	200	400	600
*Hin*d III	12	65	66	78	93	50	36
Kpn I	15	42	37	56	70	41	35
Sal I	21	94	89	100	102	95	89

(01–20 and 02–1) produced little melanin on the spore plate for seven days at 26°C under black light. There was no characteristic change after culturing for three generations. Both mutants were obtained by using *Hin*d III as the restriction enzyme. Six strains (01–4, 01–18, 01–23, 01–36, 02–19 and 02–43) of the transformants were obtained by injecting them to identified varieties. One week after injection, 01–4 and 01–36 strains showed non-affinity but wild-type M131 had affinity to some differential varieties. The strains 01–18, 01–23, 02–19 and 02–43 showed affinity but wild-type M131 had no affinity to some differential varieties (Table 2). All of the six transformants had normal growth and sporulation characteristics, and no obvious morphological differences were observed.

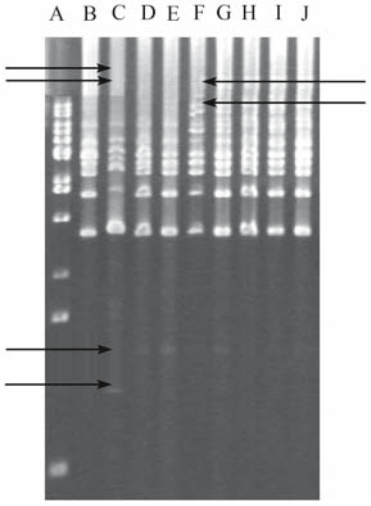
Table 2 Changes in pathogenicity of partial transformants to differential varieties of rice

Transformant	Differential variety	Resistance gene	Reaction type (M131/mutant)
01–4	F-80-1	*Pi-K*^{s}	S[a)]/R[b)]
01–18	K_{60}	*Pi-K*^{p}, *Pi-sh*	R/S
01–23	K_{3}	*Pi-K*^{h}	R/S
01–36	K_{1}	*Pi-ta*	S/R
02–19	F-129-1	*Pi-K*^{p}	R/S
02–43	F-129-1	*Pi-K*^{p}	R/S

Note: [a)] stands for sentitive; [b)] stands for resistant.

3.3 Rep-PCR analysis of mutants

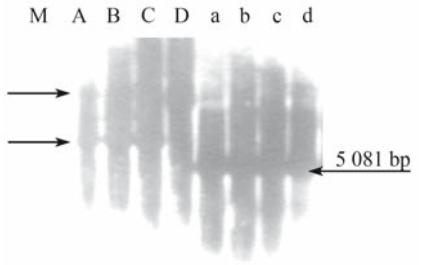
The rep-PCR fingerprint of transformants using primers *Pot*2–1 and *Pot*2–2 showed differences among the mutant and wild-type strains, especially the 01–18 and 01–36. These strains were viewed as mutants at the molecular level (Fig. 1).



A, B, C, D, E, F, G, H, I and J stand for M, M131, 01–18, 01–23, 01–4, 01–36, 02–19, 02–43, 01–20, 02–1 respectively.
Fig. 1 Rep-PCR fingerprint of transformants

3.4 RFLP analysis of mutants

Mutants 01–20, 02–1, 01–18 and 01–36 showed a strongly hybridizing 5.08 kb *Hin*d III fragment (vector size) that could have resulted from a tandem integration of the vector in one location or several independent REMIs. In southern blots of *Hin*d III and *Eco*RV digests, the two melanin mutants and the six pathogenicity mutants had nearly identical patterns. In Fig. 2, the hybridization patterns for four REMI tranformants are shown. The genome of two different strains at the molecular level, 01–18 and 01–36, and two morphologically different strains, 01–20 and 02–1, were digested with *Eco*RV and *Hin*d III and hybridized with the probe pUCATPH plasmid DNA. The results showed that the RFLP fingerprints of these transformants varied with different restriction enzymes and the plasmid was randomly inserted in all four mutants. There were two copies in the REMI transformants with *Eco*RV enzyme and one copy with *Hin*d III enzyme.



A–D: Digested with *Eco*RV; a–d: Digested with *Hin*d III; A and a: 01–18; B and b: 01–20; C and c: 02–1; D and d: 01–36
Fig. 2 RFLP fingerprint of M131 and transformants

4 Discussion

In other fungal systems, random insertional mutagenesis has been used to obtain mutants after the insertion of a plasmid into the genome. The feasibility of insertional mutagenesis for isolating deficient mutants in *M. grisea* was evaluated by transformation with the addition of restriction enzymes. It is suggested that the presence of target sites for integration, rather than the availability of foreign DNA, is the rate-limiting factor for plasmid integration. The modification of the conventional transformation procedure by the addition of restriction enzymes to the transformation mixtures (REMI) may increase both the transformation frequency and the number of single-copy integration events in many fungi and potentially randomize integration sites.

It is the modified and simple method to obtain and screen mutants with hyg B by REMI (Ellingboe, 1990) that can increase the transformation efficiency of protoplast and easily produce mutants. However, the transformation efficiency of linearized plasmid with different restriction enzymes is higher than that of circular plasmid and the increase in multiplicity is different, probably because the different restriction enzymes have different digestion sites on the genomic DNA

sequence and different recognition sites (such as methylate) with existing efficiency. The transformation efficiency is related to the dosage of enzymes and plasmids in the transformation system. The PEG was used to increase the transformation efficiency of protoplast.

It has been shown that both the linearization of plasmid pUCATPH and the presence of *Hin*d III during transformation significantly increased the transformation rate and the percentage of single-copy integrations in *M. grisea* (Table 1). Among 639 REMI transformants analyzed, two morphological mutants (01–20 and 02–1) were identified because of their low melanin production and six pathogenicity mutants (01–4, 01–18, 01–23, 01–36, 02–19 and 02–43) were identified by inoculating them to differential varieties. Rep-PCR analysis (George, 1998) showed that the two strains, 01–18 and 01–36, were much different from wild-type M131. It is suggested that the insertion sites of plasmid pUCATPH is related to the avirulence genes of *Pi–K*^{p}, *Pi–sh* resistant genes in 01–18, and *Pi–ta* resistant genes in 01–36. These insertion sites of avirulence genes can be tagged and the segments can be cloned to find the link to the molecular marker. Southern blot hybridization using the pUCATPH DNA as a probe revealed that the different phenotype is due to an integration event. Two restriction enzymes were used in RFLP. *Hin*d III is the transformation enzyme and has a site on pUCATPH. All strains with the two restriction enzymes that hybridized with plasmid pUCATPH have signal bands. Many signal bands overlapped in the 5 081 bp probably because the M131 genome has many *Hin*d III enzyme sites. There are two signal bands above 5 081 bp with the *Eco*RV enzyme because *Eco*RV has no site on pUCATPH.

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