PIRA-PCR for Detection of *Fusarium fujikuroi* Genotypes with Carbendazim-Resistance Alleles

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Abstract

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Carbendazim, a methyl benzimidazole carbamate (MBC)-group fungicide, has been used to control rice bakanae disease, caused by *Fusarium fujikuroi* (teleomorph: *Gibberella fujikuroi*), for decades in China. Previous research revealed that point mutations (E198V, GAG to GTG at codon 198, and F200Y, TTC to TAC at codon 200) of the β_2 -tubulin gene conferred resistance of *F. fujikuroi* to MBC. In this study, primer-introduced restriction analysis polymerase chain reaction (PIRA-PCR) was developed to determine genotypes with resistance of *F. fujikuroi* to MBC. A PCR template of each strain was created by an outer primer pair. Fragments with 177 bp (for mutation at codon 235) and 146 bp (for E198V) were amplified by nested PCR,

with two inner primer pairs designed and synthesized according to the nucleotide sequence of β_2 -tubulin for further enzyme digestion validation, respectively. *AccII* and *PmaCI* restriction enzyme recognition sites were introduced artificially by inner primers to differentiate MBC-sensitive and -resistant strains, respectively. The sensitivity of each strain to MBC was indirectly determined by analyzing electrophoresis patterns of the resulting amplified fragments after simultaneous digestion by both *AccII* and *PmaCI*. PIRA-PCR produced the same result as conventional methods in 6% of the time. PIRA-PCR is a sensitive and effective method for genotyping resistance alleles of *F. fujikuroi* strains to MBC.

Rice bakanae, caused by *Fusarium fujikuroi* Nirenberg (teleomorph: *Gibberella fujikuroi* (Sawada) Wollenw.), is an important disease in rice-growing regions worldwide. Among members of the *G. fujikuroi* species complex, three mating populations are associated with bakanae on rice: (i) mating type population A (anamorph *F. verticillioides* = *F. moniliforme*), (ii) mating type population C (anamorph *F. fujikuroi*), and (iii) mating type population D (anamorph *F. proliferatum*) (Amoah et al. 1995; Desjardins et al. 2000). In China, *F. fujikuroi* is the predominant species. Bakanae is a seed-borne disease which occurs during the transplanting of rice to fields. The typical symptoms of this disease are excessive elongation of seedlings or plants, or death after transplanting. Rice bakanae not only causes 10 to 50% yield damage in a year but also contaminates rice with mycotoxins produced by *F. fujikuroi* (Logrieco et al. 2003). Though seed control makes it much easier to protect rice from this disease, several studies demonstrated that the seed is not the only possible source of inoculum (Carter et al. 2008).

Before the 1970s, prevention of bakanae relied on protective fungicides, including lime and formalin. Since then, benzimidazole derivatives (methyl benzimidazole carbamates [MBC]), a group of broad-spectrum systemic fungicides, have been widely used to control this disease. Representative MBC fungicides include benomyl, fuberidazole, thiabendazole, thiophanate, and thiophanate-methyl. The repeated use of these fungicides has been widely shown to be selective pressure for resistance development (Chen et al. 2009; Irie 1988; Pan et al. 1997; Qiu et al. 2011). The first resistance case of *F. moniliforme* (synonym, *F. verticillioides*) against MBC, was reported in 1980s in Japan (Irie 1988). Populations of MBC-resistant (MBC^R) *F. moniliforme* strains were first detected in the eastern China, including Shanghai, Jiangsu, and Hebei, since 1990 (Pan et al. 1997).

Molecular mechanisms contributing to resistance against benzimidazole fungicides have been studied extensively, and substitutions of Phe167Tyr, Glu198Gly, Glu198Ala, Glu198Val, Phe200Tyr, and other codons in the β -tubulin gene have been associated with resistant phenotypes in numerous plant pathogens (Chen et al. 2009; Qiu et al. 2011). Based on the genome databases of *F. graminearum*, *F. oxysporum*, and *F. verticillioides* (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html), the tubulin gene family of each pathogen has five members, including α_1 -, α_2 -, β_1 -, β_2 -, and γ -tubulins. To form microtubulinules, the dimers of α - and β -tubulin bind to GTP and assemble onto the (+) ends of microtubulinules while in the GTP-bound state (Nogales et al. 1998). Within the *G. fujikuroi* species complex associated with rice bakanae, Yan and Dickman (1996) found that substitution of Asp50Tyr in β_1 -tubulin in *F. verticillioides* UV mutants confers cold sensitivity and benomyl resistance. Previous studies showed that a β_1 -tubulin gene (JQ026022) in *F. fujikuroi* had 99% amino acid identity with that of the MBC^R gene in *F. verticillioides* but no mutations were associated with the resistance to benzimidazole fungicides (Ma et al. 2012). This demonstrated that the resistance mechanism of *F. fujikuroi* to benzimidazole fungicides is different from that of *F. verticillioides*, though the two species belong to the same *F. fujikuroi* complex. Recent research of our laboratory showed that highly resistant strains (MBC^{HR}) of *F. fujikuroi* have two point mutations in the β_2 -tubulin gene (E198V + GGC[Gly]→GGT[Gly] at codon 235), while moderately resistant strains (MBC^{MR}) also have two point mutations in the β_2 -tubulin gene (F200Y + GGC[Gly]→GGT[Gly] at codon 235) (Chen et al. 2014). No other mutations related to benzimidazole resistance have been detected.

To maintain sound rice cultivation, it is important to locate the MBC^R populations and prevent their development. Advances in molecular biology have provided polymerase chain reaction (PCR)-based diagnosis techniques to detect fungicide-resistant genotypes in plant pathogens. Owing to single-nucleotide polymorphisms (SNP) related to resistance to MBC in *F. fujikuroi*, a method for SNP analysis is needed to detect the resistance allele. To date, several techniques for SNP detection are available, including whole-genome DNA sequencing, allele-specific (AS)-PCR (Ma et al. 2003),

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denaturing high-performance liquid chromatography (DHPLC) (Bäumler et al. 2003), PCR restriction fragment length polymorphism (RFLP) (Ma et al. 2005), pyrosequencing, quantitative real-time (qPCR), and hydrolysis probe (Saito et al. 2003). PCR-RFLP, based on introducing a restriction enzyme site caused by a point mutation, is an easy, rapid, and cheap technique to detect point mutations. A restriction enzyme recognition site, however, may be unavailable in some target DNA fragments. Under these circumstances, primer-introduced restriction analysis (PIRA)-PCR becomes a useful technique to detect point mutations (Haliassos et al. 1989; Kanae et al. 2005; Sakamoto et al. 2008). PIRA-PCR is divided into two steps, as follows: (i) introducing an artificial restriction enzyme site in a target gene, which lacks a restriction site around the mutation nucleotide (using mismatched primers); and (ii) analyzing RFLP (Haliassos et al. 1989; Luo et al. 2009).

The point mutation at the 198th codon in the β_2 -tubulin gene of *F. fujikuroi* lacks a nearby restriction enzyme site. To enhance the efficiency and sensitivity of PCR, the current study combined nested PCR and PIRA-PCR to detect carbendazim-resistant isolates of *F. fujikuroi*. This method successfully genotyped the high-carbendazim-resistance allele from sensitive and moderately and highly resistant isolates. In addition, the genotypes were obtained by analyzing the electrophoresis patterns.

Materials and Methods

Fungicides, fungal strains, and culture conditions. Technical-grade carbendazim (MBC) was generously provided by the Shenyang Academy of Chemistry and Industry (China). The fungicide was dissolved in 0.1 M hydrochloric acid (HCl) at 10 mg ml⁻¹ as a stock solution and was added to autoclaved media. The pH was adjusted to 6.8 in all potato sucrose agar (PSA) media using HCl.

In this study, 14 strains of *F. fujikuroi* were arbitrarily selected from 68 strains, which were isolated from infected rice stems of different fields in Jiangsu, following the method described previously by Carter et al. (Carter et al. 2008) (Table 1). The strains were incubated on PSA plates at 25°C. Single-spore strains were obtained and maintained at 4°C on PSA.

MBC sensitivity assay. Carbendazim stock solution at 10 mg ml⁻¹ was added to the PSA medium after sterilization to produce concentrations of 0, 0.5, 1, 2, 4, 8, and 16 µg ml⁻¹ of medium for sensitivity assays, and of 0, 5, 10, 50, and 100 µg ml⁻¹ for minimum inhibitory concentration (MIC; a concentration resulting in 100% mycelial growth inhibition) assays. To determine the sensitivity of

F. fujikuroi to carbendazim, mycelia plugs (5 mm in diameter) from the edge of a 7-day-old colony of each single-spore strain were placed on a series of PSA plates amended with MBC at each of the above concentrations for a sensitivity assay. After 25°C for 7 days, radial growth were measured, and the growth inhibition as percent of control was calculated. The values for a concentration resulting in 50% mycelial growth inhibition (EC₅₀) for each strain was calculated by regressing percent growth inhibition against the log of fungicide concentration. Each concentration has three replicates and the test was repeated twice.

DNA extraction. The 14 strains were incubated at 25°C on PSA for 7 days. Mycelia were collected into a 2-ml tube and shaken at 37°C and 175 rpm for 3 h in the presence of 600 µl of cetyltrimethylammonium bromide and 600 µl of phenol-chloroform-isopentanol. Then, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C. The upper phase was transferred into a new 2-ml tube, mixed with an equal isopropanol volume, and centrifuged at 12,000 rpm for 10 min at 4°C. The upper phase was transferred into a new 1.5-ml tube, mixed with double absolute alcohol volume, incubated at -20°C for 1 h, and centrifuged at 12,000 rpm for 10 min. The pellet was washed in 200 µl of 75% alcohol, centrifuged at 12,000 rpm for 10 min at 4°C, and finally dissolved in 30 µl of sterile water.

Design of one outer and two inner primer pairs. The outer primer pair, designed based on the sequence of β_2 -tubulin from *F. fujikuroi* strains, amplifies a partial gene of 1,496 bp in length. The forward primer β_2 -F anneals to nucleotide positions 133 to 150 (18 bp) of β_2 -tubulin, and the reverse primer β_2 -R anneals to nucleotide positions 1,611 to 1,628 (18 bp) (Table 2).

For nested PCR amplification, two pairs of inner primer were designed (i.e., PIRA-N-F1/PIRA-N-R1 and PIRA-N-F2/PIRA-N-R2) (Table 2).

The first forward primer, PIRA-N-F1, anneals to nucleotide positions 852 to 873 (22 bp) of β_2 -tubulin. The reverse primer, PIRA-N-R1, anneals to nucleotide position 1,007 to 1,028 (22 bp), in which nucleotide A was replaced by nucleotide G at nucleotide position 1,008. The mismatched primer introduces an artificial restriction site (CGCG) for restriction enzyme *AccII* in strains nonmutated at codon 235 in β_2 -tubulin. The resulting PCR product was 177 bp (Fig. 1).

The second forward primer, PIRA-N-F2, anneals to nucleotide positions 871 to 893 (23 bp) of β_2 -tubulin, in which nucleotide G was substituted by nucleotide C at position 890, and nucleotide C substituted for nucleotide T at position 892. The mismatched primer introduces an artificial restriction site (CACGTG) for restriction

Table 1. Origin, carbendazim resistance phenotype, and mutation in β_2 -tubulin gene in *Fusarium fujikuroi* strains in this study

Phenotype (n), names of isolates ^b	Origins	Codon number ^a		
		198	200	235
MBC ^S (12)		GAG (Glu)	TTC (Phe)	GGC (Gly)
YC3, YC15, YC18, YC20, YC27, YC36	Yancheng city, Jiangsu province	...	...	...
ZJ2, ZJ5, ZJ10, ZJ15	Zhenjiang city, Jiangsu province	...	...	...
JR5, JR8	Jurong city, Jiangsu province	...	...	...
MBC ^{MR} (25)		GAG (Glu)	TAC (Tyr)	GGT (Gly)
YCA, YCB, YCC, YCD, YCE, YCF, YCG, YCH, YCI	Yancheng city, Jiangsu province	...	...	...
ZJA, ZJB, ZJC, ZJD, ZJE, ZJF, ZJG, ZJH	Zhenjiang city, Jiangsu province	...	...	...
JRA, JRB, JRC, JRD, JRE, JRF, JRG, JRH	Jurong city, Jiangsu province	...	...	...
MBC ^{HR} (31)		GTG (Val)	TTC (Phe)	GGT (Gly)
YC4201, YC4202, YC4203, YC4204, YC4205, YC4206, YC4207, YC4208, YC4209, YC4210	Yancheng city, Jiangsu province	...	...	...
ZJ4201, ZJ4202, ZJ4203, ZJ4204, ZJ4205, ZJ4206, ZJ4207, ZJ4208, ZJ4209, ZJ4210	Zhenjiang city, Jiangsu province	...	...	...
JR4201, JR4202, JR4203, JR4204, JR4205, JR4206, JR4207, JR4208, JR4209, JR4210, JR4211	Jurong city, Jiangsu province	...	...	...

^a Nucleotides in parentheses indicate the particular point mutation in the given codon.

^b MBC^S, MBC^{MR}, and MBC^{HR} indicate sensitive, moderately resistant, and highly resistant to carbendazim, respectively; number in parenthesis indicates number of isolates.

enzyme *PmaCI* in strains nonmutated at codon 198 in β_2 -tubulin. The reverse primer PIRA-N-R2 anneals to nucleotide position 994 to 1,016 (23 bp). The resulting PCR product was 146 bp (Fig. 1).

PCR assay. The outer PCR primer pair β_2 -F/ β_2 -R (Table 2) was applied to amplify a fragment of the β_2 -tubulin gene from the genomic DNA of *F. fujikuroi*. PCR was conducted in a 25- μ l volume containing 4.0 μ l (0.2 mM) of dNTP mixture, 2.5 μ l (0.8 mM) of magnesium chloride, 1.0 μ l (0.4 mM) of each primer, 1.0 μ l of DNA, 0.25 μ l (1.25 U) of long and accurate (LA) *Taq* polymerase (TaKaRa Biotechnology [Dalian] Co., Ltd.), and 2.5 μ l of 10 $\times$ LA PCR buffer provided by the supplier. The cycling parameters were 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and extension at 72°C for 90 s; and a final extension at 72°C for 10 min. PCR products were electrophoresed through a 1.2% agarose gel in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer. The gels were stained with ethidium bromide and photographed under ultraviolet light in a Gel Documentation System (Bio-Rad, Inc.).

The initial PCR product, after being diluted 50-fold, was used as template DNA for nested PCR amplification using the inner primers pair PIRA-N-F1/PIRA-N-R1 and PIRA-N-F2/PIRA-N-R2 (Table 2). PCR was conducted in a 25- μ l reaction mixture containing 16.875 μ l of double-distilled H₂O, 1.0 μ l of template DNA, 0.125 μ l (1.25 U) of *rTaq* DNA polymerase (TaKaRa Biotechnology [Dalian] Co., Ltd.), 1.5 μ l (0.8 mM) of MgCl₂, 2.0 μ l (0.2 mM) of dNTP, 0.5 μ l (0.4 mM) of each primer, and 2.5 μ l of 10 $\times$ PCR buffer (TaKaRa Biotechnology [Dalian] Co., Ltd.). The PCR was conducted as follows: initial denaturing at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 56.5°C for the primer pair PIRA-N-F1/PIRA-N-R1 (64°C for primer pair PIRA-N-F2/PIRA-N-R2) for 30 s, and extension at 72°C for 10 s; and a final extension at 72°C for 10 min. PCR products were analyzed through a 4% agarose gel

electrophoresis in 0.5 $\times$ TBE buffer and purified by the AxyPrep PCR Cleanup Kit (Axygen Biosciences, China).

Molecular diagnosis of MBC^R alleles. Molecular diagnosis relied on the fact that the PCR products from different MBC^R alleles could be digested to produce different fragment lengths. The purified nested-PCR products were digested by restriction enzymes *AccII* and *PmaCI*, respectively. The 8 μ l of each PCR product was digested with 1 μ l of endonuclease (10 U μ l⁻¹) in 2 μ l of 10 $\times$ buffer M and 9 μ l of H₂O for 1 to 3 h at 37°C for *AccII* and in 2 μ l of 10 $\times$ buffer and 9 μ l of H₂O for 1 to 3 h at 65°C for *PmaCI*. Resulting products were electrophoresed through 4% agarose gels in 0.5 $\times$ TBE buffer.

Comparison between two methods for detecting MBC^R strains of *F. fujikuroi*. The 68 *F. fujikuroi* strains (including the 14 strains described above) were used to compare the results of the two methods. Sensitivity of each strain to carbendazim was determined by the conventional method (mycelial growth inhibition assay) and genotype was identified by PIRA-PCR. Based on the results of PIRA-PCR, six samples per genotype were arbitrarily chosen, then sequenced to validate whether the chosen strains had the expected resistance alleles.

Results

Sensitivity of *F. fujikuroi* strains to MBC. According to the EC₅₀ and MIC values of mycelial growth inhibition assays (Table 3), 2 of 14 strains were classified into sensitive (MBC^S), which could grow on PDA medium without carbendazim but which were completely inhibited by carbendazim at 5 μ g ml⁻¹, 2 strains were classified as moderately resistant (MBC^{MR}), which could grow quickly at 5 μ g ml⁻¹ and slowly at 50 μ g ml⁻¹ but which were completely inhibited at 100 μ g ml⁻¹, and 10 strains were classified as highly resistant (MBC^{HR}), which could grow at 100 μ g ml⁻¹ (Table 1).

Table 2. Polymerase chain reaction (PCR) primers used to identify different methyl benzimidazole carbamate (MBC)-resistant stains of *Fusarium fujikuroi*

Type	Primers	Nucleotide sequence of primer (5'→3')	Position	Size of PCR product (bp)	Purpose
Outer primer pair	β_2 -F	TGCTGATACTCGCTGACC	133–150	...	PCR templates
	β_2 -R	TTCTGCTTTGAGGGACAT	1,611–1,628	1,496	
Inner primer pair 1	PIRA-N-F1	ACGCCACTCTGTTCATTGAACCA	852–873	...	Used for enzyme digestion to classify MBC isolates
	PIRA-N-R1 ^a	GGAATCGGAAACATGTCGTCGC	1,007–1,028	177	
Inner primer pair 2	PIRA-N-F2 ^b	CCAGCTTGTCGAGAACTCCACG	871–893	...	Used for enzyme digestion to classify MBC ^{HR} isolates
	PIRA-N-R2	ATGTCGTCACGCCAGCCATAACT	994–1,016	146	

^a Primer PIRA-N-R1 was designed based on nucleotides of β_2 -tubulin in positions 1,007 to 1,028 (22 bp); nucleotide G substituted for nucleotide A at position 1,008 (underlined).

^b Primer PIRA-N-F2 was designed based on nucleotides of β_2 -tubulin in positions 871 to 893 (23 bp); nucleotide C substituted for nucleotide G at position 890 and nucleotide C substituted for nucleotide T at position 892, respectively (underlined).

Table 3. Sensitivity of *Fusarium fujikuroi* to carbendazim (methyl benzimidazole carbamate [MBC]) in vitro

Strains	Phenotype for MBC ^a	EC ₅₀ value (μ g ml ⁻¹) ^b	MIC value (μ g ml ⁻¹) ^c
YC3	S	1.08	MIC < 5
YC15	S	1.12	MIC < 5
YCA	MR	2.41	50 < MIC < 100
YCB	MR	2.36	50 < MIC < 100
YC4201	HR ^d	3.32	MIC > 100
YC4202	HR ^d	3.3	MIC > 100
YC4203	HR ^d	3.63	MIC > 100
YC4204	HR ^d	3.55	MIC > 100
YC4205	HR ^d	3.42	MIC > 100
YC4206	HR ^d	3.26	MIC > 100
YC4207	HR ^d	3.15	MIC > 100
YC4208	HR ^d	3.65	MIC > 100
YC4209	HR ^d	3.7	MIC > 100
YC4210	HR ^d	3.46	MIC > 100

^a S, MR, and HR indicate sensitive, moderately resistant, and highly resistant to MBC, respectively.

^b EC₅₀ indicates a concentration resulting in 50% mycelial growth inhibition. The value of each EC₅₀ is the mean of three replicates.

^c Minimum inhibitory concentration (MIC) is a concentration resulting in 100% mycelial growth inhibition.

^d Strains which were classified as highly resistant were mainly determined by MIC values (MIC > 100); EC₅₀ values are for reference only.

PCR and restriction analysis. An expected 1,496-bp DNA fragment was amplified from genomic DNA of all strains tested by the outer primer pair β_2 -F/ β_2 -R (Fig. 2). The first-round PCR product was then used as template for the PIRA-PCR. Two expected DNA fragments, in sizes of 177 bp and 146 bp, were obtained by PCR with the inner primer pairs PIRA-N-F1/PIRA-N-R1 and PIRA-N-F2/PIRA-N-R2 (Table 2; Fig. 3).

The mismatched primer PIRA-N-R1 introduced an *AccII* recognition site into codon 235 of β_2 -*tubulin* from MBC^S strains, which were nonmutated at codon 235 (Fig. 1). *AccII* digested the 177-bp PCR products from strains nonmutated at codon 235 into two fragments of 21 and 156 bp; however, the 21-bp fragment was too short to show on agarose gels. In contrast, *AccII* could not digest the PCR product of strains with a mutation at codon 235 (Fig. 4A).

A *PmaCI* recognition site was introduced by the mismatched primer PIRA-N-F2 into codon 198 of β_2 -*tubulin* from MBC^R strains, which were mutated at codon 198 (Fig. 1). The 146-bp PCR products were digested by *PmaCI* into two fragments of 22 and 124 bp. However, the 22-bp fragment was also too short to show up on the agarose gel (Fig. 4B).

The results of RFLP analysis after PIRA-PCR showed that the 177-bp fragments, amplified from MBC^S strains, can be digested only by *AccII* and into two fragments (21 and 156 bp), while the product of MBC^{HR} strains can only be digested by *PmaCI* into the other two fragments (22 and 124 bp). However, the PCR products of MBC^{MR} strains were not digested by either of the two restriction enzymes (*AccII* or *PmaCI*; Fig. 1 and 4).

Comparison of two methods for determination MBC^R strains of *F. fujikuroi*. The 68 *F. fujikuroi* strains (including the 14 strains tested) from Jiangsu had been assessed for MBC resistance by PIRA-PCR and by the conventional method (mycelial growth inhibition

assay). The two methods produced the same results. Of the 68 strains, 12 were divided into MBC^S strains, 25 were divided into MBC^{MR} strains, and 31 were divided into MBC^{HR} strains. Although the two methods produced the same results, the conventional method needs more than 7 days and the PIRA-PCR required approximately 10 h.

Discussion

Resistance in *F. fujikuroi* is an increasingly severe threat because of the continuing use of benzimidazoles in some areas of China, especially where the resistance has occurred. The present study provides the first PCR-based assay for identification of the carbendazim-resistance alleles of *F. fujikuroi*. The MBC^{HR} isolates could be distinguished from the MBC^{MR} strains by cleaving the 146-bp PCR products with *PmaCI*, and the MBC^S isolates could be differentiated from the MBC^{MR} or MBC^{HR} isolates by cleaving the 177-bp PCR products with *AccII* (Fig. 4).

The most common method for monitoring resistance of *F. fujikuroi* to MBC fungicides depends on the assessment of mycelial growth on a benzimidazole-amended medium (Zhou et al. 1994). It

Fig. 2. Fragment of the β_2 -*tubulin* gene, 1,496 bp in size, was amplified by the outer primer pair, β_2 -F/ β_2 -R. Lane M = 2,000-bp DNA ladder. Lanes 1–11: fragments were amplified from 11 *Fusarium fujikuroi* isolates, respectively, including YC3, YC15, YCA, YCB, YC4201, YC4202, YC4203, YC4204, YC4205, YC4206, and YC4207.

Fig. 1. Schematic presentation of primer-introduced restriction analysis (PIRA) polymerase chain reaction method to detect moderate and high resistance to methyl benzimidazole carbamate (MBC^{MR} and MBC^{HR}, respectively) in *Fusarium fujikuroi*. The underlined sequence is the primer PIRA-N-F2 and PIRA-N-R1, which introduces an artificial recognition site of *PmaCI* and an artificial recognition site of *AccII*, respectively. Bold characters in primers are mismatched nucleotides. Nucleotides in frames are the single-nucleotide polymorphism positions. The distance between restriction sites is not to scale.

is relatively labor-intensive and time consuming. Results from the conventional method cannot be obtained quickly enough to guide farmers to select suitable fungicides, especially when numerous isolates need testing. Therefore, a method such as PIRA-PCR that can quickly detect point mutations is preferred.

Compared with other methods available, PIRA-PCR possesses numerous advantages. It depends on common techniques of molecular biology and needs merely basic PCR and restriction enzyme reagents, one electrophoresis apparatus, and one thermocycler. This equipment is relatively cheap, inexpensive to maintain, and simple to manipulate. Furthermore, PIRA-PCR assays need little optimization. Although other PCR-based diagnosis techniques are available, including DNA direct sequencing, qPCR, pyrosequencing, and DHPLC (Bäumler et al. 2003), they cannot be commonly used because of the high-priced apparatus and reagent kits. AS-PCR is relatively rapid and simple; however, it is a challenge to standardize the reaction protocol, and it is not absolutely specific, which may cause misidentification of alleles (Newton et al. 1989; Pinto et al. 2006).

Introduction of primer-template mismatches is crucial to the design of successful restriction enzyme recognition sites. Because no restriction enzyme recognition sites were available around the point mutation at codon 198 of the β_2 -tubulin gene, we used a mismatched primer to introduce an artificial restriction site. Interaction between primer-template mismatches is known to reduce binding efficiency of PCR-fragment amplification in a PCR. Some template-primer mismatches at the 3'-terminal end of the primer, including C-C, G-A, and G-G, are much more refractory to extend and may even absolutely block the extension (Kwok et al. 1990; Little 1997). To introduce a suitable restriction enzyme recognition site in the current study, the primers PIRA-N-F2 and PIRA-N-R1 were designed with

mismatches that did not dramatically reduce the efficiency of the reaction. One mismatch was incorporated at the penultimate 3' base (in the primer PIRA-N-R1) in order to introduce a restriction site of *AccII* for identifying the MBC^S strains, while the two mismatches were incorporated at the second and fourth positions from 3' end (in the primer PIRA-N-F2) to create a restriction site (*PmaCI*) for identifying the MBC^{HR} strains.

According to Chen et al. (2014), mutations at codons 198, 200, and 235 in the β_2 -tubulin gene of *F. fujikuroi* isolates possess different phenotypic sensitivities to MBC. MBC^{MR} strains have two point mutants (F200Y + G235G), while MBC^{HR} strains possess two different point mutations (E198L + G235G). PIRA-PCR can only identify highly carbendazim-resistant isolates with β_2 -tubulin substitutions (E198L + G235G). Among field carbendazim-resistance populations, however, high resistance (E198L + G235G) is predominant and the moderate resistance (F200Y + G235G) is much rarer (unpublished data).

Successful PIRA-PCR depends on primer design. The mismatches should be close to the 3'-terminal end of the primer and the amount of mismatched base should be limited. *Taq* polymerase lacking reading-proof activity is recommended. To analyze agar electrophoresis patterns, 100 to 200 bp of PCR fragments is appropriate. An Internet-based PIRA-PCR designer is available (http://cedar.genetics.soton.ac.uk/public_html/primer2.html) (Ke et al. 2001).

In conclusion, single substitutions in the β_2 -tubulin gene of *F. fujikuroi* were relied on to develop a PIRA-PCR method for differentiating the MBC^{HR} allele. This accurate and rapid PCR-based diagnostic assay was standardized based on the link between the genotype and phenotype of isolates. Hence, the PIRA-PCR shows potential for use in genotyping MBC^{HR} isolates of *F. fujikuroi* from the field and will help farmers generate the most optimized antiresistance strategy.

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Fig. 3. Specific fragments for consequent digestion analysis were amplified by two inner primer pairs. Lane M = 20-bp ladder marker. Lanes 1–3: fragments of 177 bp in size from *Fusarium fujikuroi* isolates YC3, YCA, and YC4201, respectively, were amplified by the inner primer pair PIRA-N-F1/PIRA-N-R1. Lanes 4–6, fragments of 146 bp in size from *F. fujikuroi* isolates YC3, YCA, and YC4201, respectively, were amplified by the inner primer pair PIRA-N-F2/PIRA-N-R2.

Fig. 4. Restriction patterns of β_2 -tubulin fragments from *Fusarium fujikuroi* were digested by restriction enzymes. Lane M, 20-bp ladder marker. Lanes 1–3, isolates YC3, YCA, and YC4201, respectively. **A**, *AccII* digest: fragments in lane 1 are 21 and 156 bp but the 21-bp fragment is too short to show on 4% agarose gel; lanes 2 and 3 are 177 bp. **B**, *PmaCI* digest: fragments in lane 3 are 22 and 124 bp and fragments in lane 1 and 2 are 146 bp but the 22-bp fragment is too short to show on 4% agarose gel.

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