Plant Pathology (2009) **58**, 882–887 Doi: 10.1111/j.1365-3059.2009.02090.x

PIRA-PCR for detection of *Fusarium graminearum* genotypes with moderate resistance to carbendazim

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PIRA-PCR (primer-introduced restriction analysis PCR) was developed to detect isolates of *Fusarium graminearum* with moderate resistance to carbendazim, a methyl benzimidazole carbamate (MBC)-group fungicide. Two primer pairs were designed and synthesized according to the nucleotide sequence of the β_2-tubulin gene from *F. graminearum*. Fragments of 164 bp were amplified by nested PCR from isolates differing in carbendazim sensitivity. A *Hin*dIII restriction enzyme recognition site was introduced artificially by inner primers to detect a mutation at codon 167, and *Taa*I (*Tsp*4CI) restriction enzyme was used to detect a mutation at codon 200. The sensitivity of isolates to carbendazim was determined by analyzing electrophoresis patterns of the resulting PCR products after simultaneous digestion with both *Hin*dIII and *Taa*I. Results from PIRA-PCR and a conventional method (mycelial growth on agar) were identical but PIRA-PCR required only 7–8 h while the conventional method required 5–7 days. This study demonstrates that PIRA-PCR not only monitors the appearance of moderately resistant isolates, but can be useful for detecting genotypes of *F. graminearum* with moderate resistance to carbendazim.

Keywords: *Gibberella zeae*, methyl benzimidazole carbamates (MBC), molecular detection, single nucleotide mutation, wheat scab, β_2-tubulin

Introduction

Fusarium head blight (FHB) or scab caused by *Fusarium graminearum* (sexual stage, *Gibberella zeae*) is an economically important disease on small grain cereal crops worldwide and is especially serious in China. Over the past three decades, methyl benzimidazole carbamate (MBC) fungicides, particularly carbendazim, have been relied on to control FHB in China. However, in recent years epidemics of FHB in China have become more severe and frequent (Bai & Shaner, 1994; Yao & Lu, 2000; Lu *et al*., 2001). The resistance of *F. graminearum* to carbendazim fungicides often develops under high pressure of FHB and fungicide use, leading to serious economic loss (Wang *et al*., 2002).

Resistance to MBC fungicides has been detected in many fungal species. In most cases, resistance is correlated with point mutations in the β-tubulin gene, which results in altered amino acid sequences at the benzimidazole-binding site (Ma *et al*., 2005). However, the mechanism of resistance to carbendazim in *F. graminearum* is different (Li *et al*., 2003; Chung *et al*., 2008). Previous studies show that resistance of *F. graminearum* to carbendazim is mainly caused by point mutations in the β_2-tubulin gene (FGSG_06611·3) (Chen, 2004; Chen *et al*., 2008). The results reveal that alterations either at amino acid codons 167 or 200 in β_2-tubulin (phenylalanine converted to tyrosine at both codons; TTT → TAT at codon 167, TTC → TAC at codon 200; F167Y, F200Y) are linked to moderate carbendazim resistance.

To predict the increase of fungicide resistance and to use fungicide and other disease control tactics effectively, researchers must develop methods to detect resistance early. Because of single nucleotide polymorphisms (SNPs) associated with resistance to the MBC-group fungicides in *F. graminearum*, a method for SNPs analysis is needed to detect mutations related to resistance. There are several common techniques for SNPs analysis, such as DNA direct sequencing, allele-specific PCR (AS-PCR; Ma *et al*., 2003), denaturing high performance liquid chromatography (DHPLC; Baumler *et al*., 2003), PCR-restriction fragment length polymorphism (PCR-RFLP; Ma *et al*., 2005), and TaqMan-MGB (Saito *et al*., 2003). PCR-RFLP, which is based on the alteration of a restriction enzyme site caused by a point mutation, is a rapid, easy, and cost-effective technique to detect point mutations. However, some target DNA fragments may lack a restriction enzyme recognition sequence. In these cases, primer-introduced restriction analysis PCR (PIRA-PCR) becomes a useful method to detect single nucleotide mutations (Haliassos *et al*., 1989; Kanae *et al*., 2005; Sakamoto *et al*., 2008). PIRA-PCR first introduces an artificial restriction site in

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Published online 16 June 2009

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Table 1 The origin and phenotype (sensitivity to carbendazim) of *Fusarium graminearum*, and the correlation between phenotype and the mutant of β_2-tubulin[a] in this paper

Phenotype[b]	Number of isolates	Names of isolates	Origin	Codon number[c]	
				167	200
C^S	2	ZF-43, ZF-21	Haining City, Zhejiang Province	TTT	TTC
C^S	10	S1–S10	Tongzhou City, Jiangsu Province		
C^{MR}	2	ZF-52, ZF-54	Haining City, Zhejiang Province	T[A]T	TTC
C^{MR}	10	R1–R10	Jiangsu Province		
C^{MR}	2	NT-7, t1	Jiangsu Province	TTT	T[A]C

[a]The correlation between phenotype and the mutant of β_2-tubulin is according to Chen *et al.* (2008) and sequence of β_2-tubulin gene.
[b]C^S and C^{MR} indicate sensitivity and moderate resistance to carbendazim, respectively.
[c]Location of amino acid with mutant nucleotide in β_2-tubulin. Characters in frames are mutant nucleotide.

any gene lacking a restriction site near the mutation nucleotide (using primers containing mismatches) and then uses restriction fragment length polymorphism (RFLP) analysis (Haliassos *et al.*, 1989; Ke *et al.*, 2001).

The point mutation at codon 167 in the β_2-tubulin gene of *F. graminearum* lacks a nearby restriction enzyme recognition site. To increase the sensitivity and efficiency of PCR, the current study combined PIRA-PCR and nested PCR to detect carbendazim-resistant isolates of *F. graminearum*. This method successfully differentiated between moderately resistant and sensitive isolates. In addition, the genotypes were obtained by analyzing the electrophoresis patterns.

Materials and methods

Fungal isolates

A total of 26 isolates of *F. graminearum* were collected from infected wheat spikelets originating from different fields in Jiangsu and Zhejiang Provinces of China (Table 1). The isolates were incubated on potato dextrose agar (PDA) plates at 25°C. Single-spore isolates were obtained and maintained at 4°C on PDA.

Carbendazim susceptibility assay

Carbendazim was provided by the Shenyang Academy of Chemistry and Industry (China). The fungicide was dissolved in 0·1 M hydrochloric acid (HCl) and adjusted to 10 mg mL^{-1}. Carbendazim was added to the PDA medium after sterilization to produce concentrations of 0, 1·4, 50 or 100 $\mu g\,mL^{-1}$ medium. To determine the sensitivity of *F. graminearum* to carbendazim, one 5-mm mycelial plug taken from the edge of a 3-day-old colony of each single-spore isolate was placed on PDA plates amended with carbendazim at each of the above concentrations. There were three replicate plates for each concentration, and the experiment was performed twice. After the plates had been incubated at 25°C for 5 days, radial growth was assessed. According to previous studies (Yuan & Zhou, 2005; Chen *et al.*, 2007), the isolates were classified into three phenotypes: sensitive isolates (C^S, which could grow on PDA medium without carbendazim but which were completely inhibited by 1·4 $\mu g\ mL^{-1}$ carbendazim); moderately resistant isolates (C^{MR}, which could grow quickly at 1·4 $\mu g\,mL^{-1}$ and slowly at 50 $\mu g\,mL^{-1}$ but which were completely inhibited at 100 $\mu g\ mL^{-1}$); and highly resistant isolates (C^{HR}, which could grow at 100 $\mu g\,mL^{-1}$).

DNA extraction

The 26 isolates were grown at 25°C on PDA for 5 days. Mycelia were harvested and ground to a fine powder in the presence of liquid nitrogen for DNA extraction. DNA was extracted and purified according to the CTAB method of Nicholson & Parry (1996). Using the methods described by Edwards *et al.* (2001), 50 samples of DNA were also extracted from the diseased wheat spikelets that had been collected for isolation of *F. graminearum*.

Design of inner and outer primers

The outer primers were created based on *F. graminearum* DNA for β_2-tubulin (FGSG_06 611·3) according to the position of point mutations in resistant isolates. The forward primer NoF anneals to nucleotide positions 657 to 675 (19 bp) of β_2-tubulin, and the reverse primer NoR anneals to nucleotide positions 991 to 1012 (22 bp); the resulting PCR product is 356 bp (Table 2).

For nested PCR amplification, a pair of inner primers was designed. The forward primer Hind3F anneals to nucleotide positions 793 to 812 (20 bp) of β_2-tubulin, in which nucleotide A substituted for nucleotide C at position 808, and nucleotide G substituted for nucleotide C at position 810. The mismatched primer creates an artificial restriction site (AAGCTT) for restriction enzyme *Hin*dIII in isolates non-mutated at codon 167 in β_2-tubulin. The reverse primer Hind3R anneals to nucleotide position 933 to 956 (24 bp). The resulting PCR product is 164 bp (Table 2).

PCR assay

The outer PCR primer pair NoF + NoR (Table 2) was used to amplify a fragment of the β_2-tubulin gene from the genomic DNA of *F. graminearum*. The PCR reactions were conducted in a 50-μL volume containing 0·4 mM of

Table 2 Nucleotide sequence of primers used in PCR amplification of fragments of the β_2-tubulin gene from *Fusarium graminearum*

Name of primer	Nucleotide sequence of primer	PCR product
NoF	5′ AAGCCATTGATGTTGTTCG 3′	356 bp
NoR	5′ CATGACGGTAGAAATCAGGTAG 3′	
Hind3F[a]	5′ CGATCGCATAATGGC**A**A**G**CT 3′	164 bp
Hind3R	5′ GGGTCCTCTCGTAGATATCGTACA 3′	

[a]Primer Hind3F was designed from nucleotides of β_2-tubulin in positions 793–812 (20 bp); nucleotide A substituted for nucleotide C at position 808 and G substituted for C at position 810.

each dNTP, 1·5 mM magnesium chloride, 0·2 μM of each primer, 1 × PCR buffer and 1·25 U *Taq* Polymerase.

The PCR amplifications were carried out in a PTC 200 thermocycler (MJ Research, Inc.) using the following parameters: initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 5 min. PCR products were electrophoresed through a 2% agarose gel in 0·5 × TBE buffer. The gels were stained with ethidium bromide and photographed under ultra-violet light in a Gel Documentation System (Bio-Rad, Inc.).

After being diluted 50 times, the resulting PCR product was used as template DNA for nested PCR amplification using the inner primer pair Hind3F + Hind3R (Table 2). The PCR reaction mixtures were the same as for the first PCR amplification. The thermocycling conditions for the nested PCR were 94°C for 5 min; followed by 35 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 5 min. PCR products were electrophoresed through a 2% agarose gel in 0·5 × TBE buffer and purified by the AxyPrep PCR Cleanup Kit (Axygen, Inc.).

Molecular detection of carbendazim-resistant isolates

Molecular diagnostic assays were based on the fact that the PCR products from different alleles could be digested to produce different patterns (different fragment lengths) by a restriction enzyme. The purified PCR products obtained by the second round of PCR amplification were digested by restriction enzymes *Hin*dIII (MBI Fermentas) and *Taa*I (*Tsp*4CI; MBI Fermentas). Eight microlitres of PCR product was digested with 1 μL endonuclease (10 U μL^{-1}) in 2 μL 10 × buffer R and 9 μL H_2O for 1–3 h at 37°C for *Hin*dIII and in 2 μL 10 × buffer Tango and 9 μL H_2O for 1–3 h at 65°C for *Taa*I. Digests were analyzed on 3% agarose gels in 0·5 × TBE buffer.

Comparison of two methods for detecting carbendazim-resistant isolates of *F. graminearum*

A total of 50 random samples of infected kernels were collected from Jiangsu Province. One isolate of *F. graminearum* was obtained from each sample, and its sensitivity to carbendazim was tested by the conventional method (growth on carbendazim-amended PDA, as described earlier) and by PIRA-PCR. According to the results of PIRA-PCR, three samples per genotype were randomly selected and then sequenced to validate that the selected isolates had the expected genotypes.

Results

Susceptibility of *Fusarium graminearum* isolates to carbendazim

Based on radial growth on PDA amended with 0, 1·4, 50 or 100 $\mu g\ mL^{-1}$ of carbendazim, 12 of the 26 isolates were classified as sensitive (C^S) and 14 were classified as moderately resistant (C^{MR}) (Table 1). None of the isolates were highly resistant.

PCR analysis

As expected, the outer primer pair NoF + NoR amplified a 356 bp DNA fragment from genomic DNA of *F. graminearum* in the first round of PCR. The first-round PCR product then served as template for the PIRA-PCR. An expected 164 bp DNA fragment was obtained by PCR with the inner primer pair Hind3F + Hind3R.

Restriction analysis

The final PCR product amplified by primer pair Hind3F + Hind3R was digested by restriction enzyme *Hin*dIII and *Taa*I. The mismatched primer Hind3F introduced a *Hin*dIII recognition site into the PIRA-PCR product of isolates non-mutated at codon 167 (Fig. 1). The *Hin*dIII digested the 164 bp PCR product from isolates non-mutated at codon 167 into two fragments of 16 and 148 bp, but the 16 bp fragment was too short to show on agarose gels. In contrast, the enzyme did not digest the PCR product of isolates with a mutation at codon 167 (Fig. 2a). A *Taa*I recognition site exists in the 164 bp fragment from all isolates, but one more recognition site (ACTGT) exists in the isolates with a mutation at codon 200 (TTC → TAC) (Fig. 1). With *Taa*I, the 164-bp PCR product of isolates with a mutation at codon 200 was digested into 42, 55, and 67 bp fragments, while the PCR products of other isolates were digested into 55 and 109 bp fragments (Fig. 2b).

The results of restriction analysis by PIRA-PCR showed that the PCR product of carbendazim-sensitive isolates can be digested by *Hin*dIII and into two fragments (55 and 109 bp) by *Taa*I, while the product of carbendazim-resistant isolates mutated at codon 167 (C^{MR}) can only be digested by *Taa*I. The PCR products of resistant isolates mutated at codon 200 (C^{MR}) can be digested into three fragments (42, 55 and 67 bp) by *Taa*I (Fig. 2).

Comparison of two methods for detecting carbendazim-resistant isolates of *F. graminearum*

Fifty randomly selected *F. graminearum* isolates from Jiangsu Province were tested for carbendazim resistance

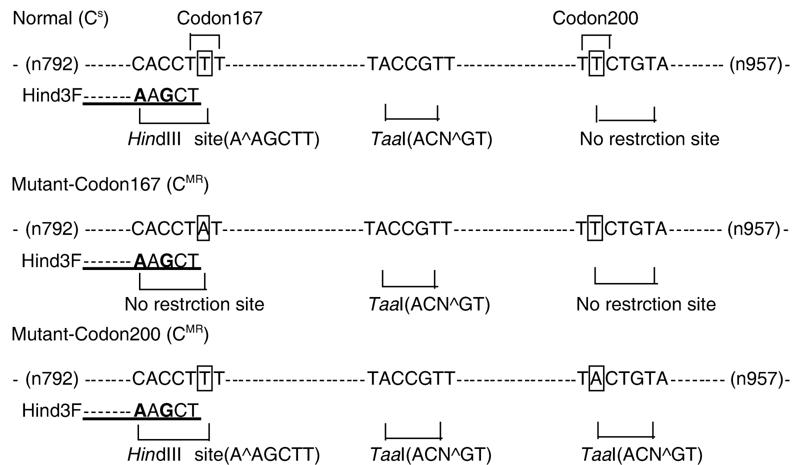

Figure 1 Schematic presentation of primer-introduced restriction analysis (PIRA)-PCR method to detect moderate resistance to carbendazim in *Fusarium graminearum*. The underlined sequence is the primer Hind3F, which introduces an artificial *Hin*dIII recognition site. Primer characters in bold are mismatched nucleotides. Characters in frames are the SNP position. The distance between restriction sites is not to scale.

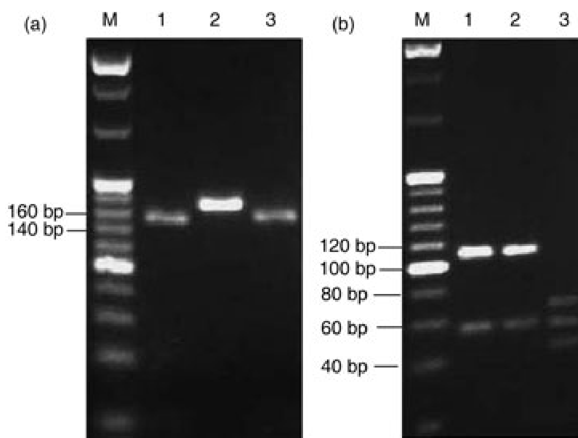

Figure 2 Restriction patterns of β_2-tubulin fragments from *Fusarium graminearum* digested by restriction enzymes. M, 20 bp marker. Lanes 1–3, isolates ZF43, ZF52 and NT-7, respectively. a), *Hin*dIII digest: fragments in lanes 1 and 3 are 16 and 148 bp, but the 16-bp fragment is too short to show on 3% agarose gel; lane 2 is 164 bp. b), *Taa*I digest: fragments in lanes 1 and 2 are 55 and 109 bp, and fragments in lane 3 are 42, 55 and 67 bp.

Table 3 Comparison of two methods for detecting carbendazim resistance in *Fusarium graminearum* isolates

Method	Number of isolates tested	Number of C^{MRa}	Number of C^{S}	Detection Period
Mycelial growth test	50	14	36	5–7 days
PIRA-PCR[b]	50	14	36	7–8 hours

[a]C^S and C^{MR} are sensitive and moderately resistant to carbendazim, respectively.
[b]Primer-introduced restriction analysis PCR.

Table 4 The genotype of *Fusarium graminearum* as determined by primer-introduced restriction analysis (PIRA)-PCR

Phenotype[a]	Number of isolates	Codon number[b] 167	200
C^S	36	TTT	TTC
C^{MR}	10	TAT	TTC
C^{MR}	4	TTT	TAC

[a]C^S and C^{MR} are sensitive and moderately resistant to carbendazim, respectively.
[b]Location of amino acid with mutant nucleotide in β_2-tubulin. Characters in frames are mutant nucleotide.

by the conventional method (growth on fungicide-amended agar) and by PIRA-PCR. As indicated in Table 3, the two methods produced identical results. Out of 50 isolates, 14 were classified as moderately resistant (C^{MR}). Whilst the conventional method required more than 5 days, PIRA-PCR required less than half a day. Moreover, the genotypes of the isolates were obtained from the PIRA-PCR (Table 4). Ten moderately carbendazim-resistant isolates were identified as the β_2-tubulin variant F167Y and four as F200Y. Three samples randomly selected from each genotype were sequenced. The sequence results agreed with the restriction analysis (Table 4).

Discussion

In this study, the level of resistance to carbendazim by *F. graminearum* isolates was determined by a conventional method and by the PIRA-PCR method. The results clearly demonstrate that PIRA-PCR is rapid and effective in detecting moderate resistance of the fusarium head blight fungus to carbendazim. The results also demonstrate that PIRA-PCR correctly genotypes the mutation codons in the β_2-tubulin gene.

The most commonly used method for monitoring resistance of *F. graminearum* to MBC fungicides is based on the measurement of mycelial growth on PDA plates amended with the fungicide (Zhou *et al*., 1994). This conventional method is relatively labour-intensive and time-consuming. Results from the conventional method cannot be obtained rapidly enough to guide growers on selection of fungicides, especially when large numbers of

isolates require testing. Thus, a method like PIRA-PCR that can rapidly detect point mutations is advantageous.

Compared with other available methods, PIRA-PCR has considerable advantages. It is based on simple techniques of molecular biology and requires only basic PCR and enzymatic restriction reagents, a thermocycler, and an electrophoresis apparatus. The equipment is relatively low priced, easy to acquire, and inexpensive to maintain. Additionally, PIRA-PCR assays are robust, requiring little optimization. Although the techniques of DNA direct sequencing, DHPLC and TaqMan-MGB are more sensitive or accurate, they cannot be routinely used because of their high cost. AS-PCR is relatively simple and rapid, but it is difficult to standardize the assay protocol and not completely specific, and can even lead to misidentification of alleles (Newton *et al*., 1989; Pinto *et al*., 2006).

Because there is no restriction enzyme recognition site around the point mutation at codon 167 of the β_2-tubulin gene, an artificial restriction site is introduced by designing a mismatched primer. Primer-template mismatches are known to reduce binding energy and the efficiency of product amplification. Some primer-template mismatches at the 3′-end of the primer, such as G-A, G-G, and C-C, are more refractory to extend and may even completely inhibit extension and abolish amplification (Kwok *et al*., 1990; Little, 1997). An adjacent mismatched base near the 3′-end will also increase the refractory effect (Kwok *et al*., 1990; Cha *et al*., 1992). To introduce an appropriate restriction enzyme recognition site, the primer Hind3F was designed with two mismatches, which can strongly reduce the efficiency of amplification. However, a nested PCR step such as that used by Jacobson & Moskovits (1991) and Kaku *et al*. (2003) was used to ensure the reliability of acquiring the PCR product with the primer-introduced *Hin*dIII recognition site.

Primer design is critical for successful PIRA-PCR. The mismatched bases should be near the 3′-end of the primer, and the base amount should be limited. For analysis of the profiles with agar electrophoresis, the length of PCR products should be 100–200 bp. Primers for PIRA-PCR can be designed on a web site (Ke *et al*., 2001). Another requirement is that only *Taq* polymerase lacking proof reading activity can be used for PIRA-PCR (Newton *et al*., 1989; Kanae *et al*., 2005).

According to Chen *et al*. (2008), isolates of *F. graminearum* mutated at amino-acid codons 167, 198 or 200 in the β_2-tubulin gene have different phenotype sensitivities. A point mutation (F167Y or F200Y) leads to a moderate resistance level to carbendazim, and another mutation (GAG → CTG, E198 L) results in a high resistance level. PIRA-PCR can only identify moderately carbendazim-resistant isolates with β_2-tubulin variants F167Y or F200Y in this study. However, among field carbendazim-resistance isolates, moderate resistance (F167Y and F200Y) is dominant and the mutation at codon 198 is rare (unpublished data).

In conclusion, single nucleotide changes in the β_2-tubulin gene permitted the development of a PIRA-PCR method that distinguished moderately carbendazim-resistant from sensitive isolates of *F. graminearum*. This rapid and precise molecular diagnostic assay was standardized according to the correlation between the phenotype and genotype of isolates. PIRA-PCR can also be adapted to work with new SNPs and can be improved by combining with direct sequencing to detect unknown mutations. Thus, PIRA-PCR has a potential application in management of carbendazim resistance in *F. graminearum* and will help researchers and growers select the best antifungal strategy to use in the field.

Acknowledgements

This study was supported by the state 973 and 863 programs from the Ministry of Science and Technology of China (No. 2006CB101907 and 2008AA10Z414) and by the Natural Science Foundation of China (No. 30671384). Some work was supervised by Dr Derek Hollomon from the University of Bristol, UK.

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