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Full Length Research Paper

Cloning of a carbendazim-resistant gene from *Colletotrichum gloeosporioides* of mango in South China

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Mango anthracnose caused by *Colletotrichum gloeosporioides* is an important disease and prevalent in tropical regions of China. High carbendazim (MBC)-resistant field strains were tested and collected. The fragments of *tub*2 were cloned, sequenced, and alignments were carried out between MBC-resistant and wild-type strains of *C. gloeosporioides*. The results showed that the amino acids were altered at residues 181,198, 237 and 363. All of the mutant positions were detected by allele-specific PCR. The allele-specific fragments were amplified in MBC-resistant strains by the positive primers but not in wild-type strains. On the contrary, the allele-specific fragments were amplified in wild-type strains by the negative primers but not in MBC-resistant strains. The preliminary findings proved that the point mutation occurred at amino acid codon 198 causing a change from glutamic acid (GAG) to alanine (GCG), which is closely associated with conferring MBC-resistance in the field. An enzyme assay was employed to further test the above results. It involved an *Acc* restriction site (CGCG) at the positions of the amino acid residues at 197 and 198 (GACGAG→GACGCG) in MBC-resistant strains, in which *Acc* digested a 329 bp fragment into 107 and 222 bp, while the fragments from wild-type strains remained undigested. Based on the above assays, all of the MBC-resistant and wild-type strains were detected successfully. It strongly suggested that the altered amino acid residue at position 198 played the leading role in conferring MBC-resistance in Mango anthracnose in south China.

Key words: *Colletotrichum gloeosporioides*, Mango, MBC-resistant gene, allele-specific PCR, enzyme assay, detection.

INTRODUCTION

Anthracnose caused by *Colletotrichum gloeosporioides* is an important disease of Mango and prevalent in tropical regions of the world. *C. gloeosporioides* can cause pre-harvest blossom blight resulting in fruitset reduction and the quiescent infection results in post-harvest losses (Dodd et al., 1991; Donkin and Oosthuyse, 1996). These quiescent infections are important since disease symptom development initiated as fruit begins to ripen on market after shipment. However, pre-harvest controls can reduce latent inoculation, as well as post-harvest fruit rot (Jeffries et al., 1990).

Mango anthracnose is commonly controlled by benzimidazole fungicides such as carbendazim, benomyl, thiophanate-methyl and thiabendazole (Dodd et al., 1991; Donkin and Oosthuyse, 1996). Benzimidazole fungicide acts as an antimitotic agent, binding to the ß-tubulins (Davidse, 1986). A systematic study of the molecular mechanism of benzimidazole tolerance had been carried out in some plant pathogens and it was concluded that amino acids 198 and 200 of ß-tubulin are important for benzimidazole binding, and mutation in any of these sites leads to benzimidazole insensitivity (Fujimura et al., 1990; Koenradt et al., 1993; Yarden and Katan, 1993; Buhr et al., 1994; Koenraadt et al., 1992; Jung et al., 1992). However, when comparison of *tub*2 was carried

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out between resistant and wild-type strains, several mutant positions exists. In recent years, there were some assays of detec-tion reported, such as site-directed mutagenesis and transformation (Mukherjee et al., 2003; DoDongsheng et al., 2004), allele-specific PCR assay (Newton et al., 1989; Imyanitov et al., 2002; Li and Zhou, 2004; Sierotzki and Gisi, 2003; Li et al., 2002), and enzyme assay (Li et al., 2002).

In this study, we use allele-specific PCR and enzyme assays to detect which single base point mutation confers MBC-resistance. The allele-specific PCR methodology is based on the principle that completely matched oligonucleotides are more efficiently used in amplifying a target sequence than a mismatched oligonucleotide primer. Under strictly controlled PCR conditions, perfectly matched primer pairs results in the amplification in the target sequences while mismatched primer pairs do not result in amplification. Interpretation of allele-specific PCR results is based on the presence or absence of specific amplified DNA fragment. Secondly, the enzyme assay relies on the principle that the mutant position creates a restriction site in resistant strains, but absent in wild-type strain's allele. So a fragment including amino acid 198 can be digested into two fragments in mutant strains, but remains undigested in wild-type strains.

In 2001, some high resistant field strains were obtained from Zhanjiang of GuangDong Province as well as Haikou and Danzhou of Hainan Province in China by *in vitro* sensitive test using PDA culture medium containing various dosages of carbendazim. The ß-tubulin genes (*tub*2) were fully cloned and sequenced from resistant and wild-type strains, and alignment was carried out to find the single amino acid mutant. Then the allele-specific PCR and enzyme assays were employed to detect whether these mutations confer MBC-resistance in *C. gloeosporioides* of Mango in Chinese tropical region.

MATERIALS AND METHODS

Fungal isolates

Three MBC-resistant mutants, ZR51, ZR43, ZR46, and two wild-type strains, ZS44 and ZS29, were obtained from Zhanjiang G.D. China. Previous studies showed the minimal inhibitory concentration (MIC) of wild-type strains was from 0.110 to 0.130 µg/ml (Zhan et al., 2005).The resistant strains were able to grow well even at a MBC concentration of 1000 µg/ml, and there was no intermediate resistant type.

Cloning and sequencing of the β-tubulin gene

The total DNA isolated from MBC-resistant mutants and wild-type strains of mango were used as templates in PCR amplification using consensus oligonucleotide primers designed according to the published sequence of ß-tubulin-encoding gene (*tub*2) of *C. gloeosporioides* f.sp. *aeschynomene* (ACCESSION:U14138#M90977). Two 20-mer oligonucleotide designated primer $P_{3\text{-}1}$ (5'-CCT ATC CTC GGT CAA GCC CA-3') and primer $P_{3\text{-}2}$ (5'-GAA GCC CAT GTT CTG GCA AA-3') were chosen on the basis of the sequence alignments. All amplifications were performed for 30 cycles with 94°C for 1 min; 58°C for 30 s; and 72°C for 3 min. PCR products were separated by agarose gel electrophoresis and cloned into pMD18-T Vector. The full length of ß-tubulin gene was sequenced in TaKaRa Biotechnology (Dalian China) Co., Ltd.

All of the *tub*2 of Mango anthracnose was aligned with existing homologs from *C. gloeosporioides* f.sp. *aeschynomene* .Then, the *tub*2 from resistant strains was aligned with wild-type strains of Mango using DNAssist software.

Allele-specific PCR assay for detection

Allele-specific primers (Table 1) were designed to have the same oligonucleotide except for the base at 3' end, which differs in this allele between mutant and wild-type strains (Newton et al., 1989; Imyanitov et al., 2002; Li and Zhou, 2004; Sierotzki and Gisi, 2003; Li et al., 2002). The PCR product of 100 to 200 bp was amplified by these primers. According to allele-specific PCR assay, the predicted allele-fragments can be amplified by positive primers in all resistant strains, but did not in the wild-type strains, whereas, the allele-fragments can be amplified by negative primers in the wild-type strains, but did not in the resistant strains.

Enzyme assay

Some single base mutations in the sequence of *tub*2 resulted in amino acid changes and potentially created an enzyme restriction at mutant position. In this study, we found an amino acid substitution at residue 198 in all resistant strains, and it created an *Acc* restriction sites in *tub*2 but not in wild-type strains. A specific fragment including amino acid residue 198 codon, was amplified and only one *Acc* site in this fragment could be digested into two fragments, which can be visualized by agarose gel electrophoresis.

RESULTS

Cloning and sequencing of ß-tubulin gene

All the fragments of resistant and wild-type strains were cloned and sequenced. The fragments were cloned into T-Vector and identified by PCR assay. All the fragments were about 2 kb, and the coding region of the ß-tubulin gene was identified by comparing the predicted amino acid sequence with the published sequences of *tub*2 in *C. gloeosporioides* f.sp. *aeschynomene* (GenBank ACCESSION: U14138#M90977). The ß-tubulin gene of *C. gloeosporioides* in Mango was designated *tub2*, since it was most similar to *tub2* of *C. gloeosporioides* f.sp. *aeschynomene*. The *tub2* of *C. gloeosporioides* in Mango had 1995 bp including 6 introns. The coding sequence had 1344 bp and deduced 447 amino acids, which were more than 99% homologous to *tub*2 of *C.gloeosporioides* f.sp. *aeschynomene*. Besides, the amino acid sequence resembled those of other fungal ß-tubulin. It is 99% identical to that of *tub2* of *C. graminicola*, *Glomerella graminicola (*exclusion of one or two different amino acid), and 98% identical to that of *tub2* of *Gibberella zeae* PH-1 and *Neotyphodium coenophialum* according to NCBI BLAST results.

Table 1. Altered amino acid of ß-tubulin in MBC-resistant compared to wild-type strains.

Isolate	Amino acid positions				
	181	198	237	363	371
Aes	E	E	T	M	A
MBC^S-ZS44	E	E	T	M	S
MBC^S-ZS29	E	E	T	M	S
MBC^R-ZR51	E	A	T	M	S
MBC^R-ZR43	E	A	T	L	S
MBC^R-ZR46	K	A	A	L	S

MBC^R-ZR51, MBC^R-ZR43, and MBC^R-ZR46 are MBC-resistant strains. MBC^S-ZS44 and MBC^S-ZS29 are wild-type strains. *Aes* represents *C. gloeosporioides* f.sp. *aeschynomene* (GenBank ACCESSION:U14138#M90977).

Table 2. Primer pairs used for allele-specific PCR and sizes of predicted products.

Mutant points	Primers	Sequence	cDNA position	Predicted fragments (bp)	Notes
181	ASP-a-1	5'-GGTCTCCGACACCGTTGTCA -3'	520-541	170	positive
	ASP-b-1	5'-GGTCTCCGACACCGTTGTCG -3'	520-541	170	negative
	Con-1	5'-GCGACCTGAACCACCTGGTCTC-3'	668-689		common
198	ASP-a-2	5'-GCCTCGTTGTCAATGCAGAAGGTCT-3'	617-593	133	positive
	ASP-b-2	5'-GCCTCGTTGTCAATGCAGAAGGTCG-3'	617-593	133	negative
	Con-2	5'-GTCGACCAGGTTCTCGATGTTG-3'	485-506		common
237	ASP-a-3	5'-TGCTGTTATGTCCGGTGTCG -3'	690-709	130	positive
	ASP-b-3	5'-TGCTGTTATGTCCGGTGTCG -3'	690-709	130	negative
	Con-3	5'-TTCATGGTCGGCTTCGCTCCCTG -3'	796-819		common
363	ASP-a-4	5'-CATTCCTCCCCGCGGCCTCAAGT-3'	1065-1087	127	positive
	ASP-b-4	5'-CATTCCTCCCCGCGGCCTCAAGA-3'	1065-1087	127	negative
	Con-4	5'-CCGTCGCAAGGCTTTCTTGCATTGG-3'	1167-1191		common

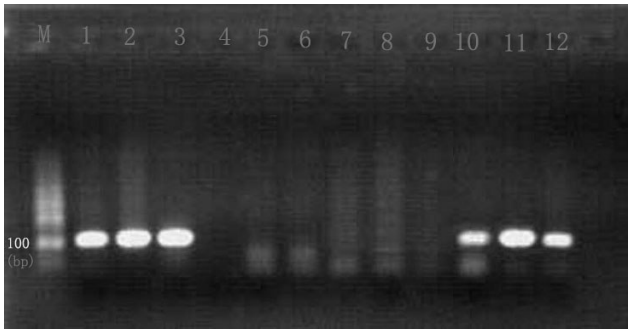

Figure 1. Allele-specific PCR analysis of MBC-resistant and wild-type allele at amino acid residue 198. Lane M: Marker DL2000; lanes 1, 2, 3, 4, 5 and 6: amplified by primers of ASP-a-2 and con-2. Lanes 7, 8, 9, 10, 11 and 12: amplified by primers of ASP-b-2 and con-2. 1, 2, 3, 7, 8, 9: resistant strains. 4, 5, 6, 10, 11 and 12: wild-type strains. Allele specific products are visible in lanes 1, 2, 3, 10, 11 and 12.

Alignment of *tub2* between mutant and wild-type strains

The single base point mutation which resulted in deduced amino acid altered was observed at points of 541, 593, 709 and 1077 in *tub*2 cDNA, with deduced amino acid at residues 181, 198, 237 and 363 based on the alignment between MBC-resistant and wild-type strains. However, at amino acid residues 181, 237 and 363, only some of resistant strains were altered at these positions, while all of the resistant strains were altered at 198 (Table 1).In this position, Ala appears in resistant strains, but Glu in wild-type strains, including in *tub2* of *C. gloeosporioides* f. sp. *aeschynomene*.

Detection by allele-specific PCR assay

Three resistant and three wild-type strains were arbitrarily employed for this detection. Allele-specific PCR assay was carried out by the specific primers as shown in Table 2.The results showed that there were no regulated visible products at amino acid residue 181, 237 and 363 detection, while at residue 198, all of well-regulated allele specific products were amplified by the positive primers in all of the resistant strains but invisible in all of the wild-type strains. In contrast, allele specific fragments were amplified by the negative primer in all wild-type strains, but not in all of the resistant strains (Figure 1).

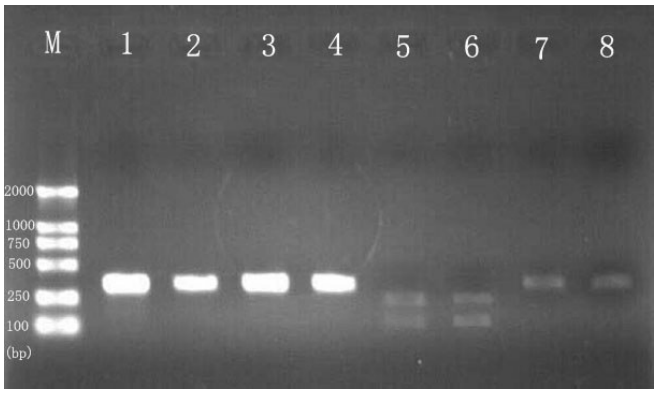

Figure 2. The map of digested by *Acc*I for detection of mutant isolates. Lane M: Marker DL2000; lanes 1 and 2: 329 bp fragments amplified from *tub*2 of ZR51 and ZR46; lanes 3 and 4: 329 bp fragments amplified from *tub*2 of ZS44 and ZS29; lanes 5 and 6: digested fragments from ZR51 and ZR46 into 107 and 222 bp by *Acc*I; lanes 7 and 8: fragments from ZS44 and ZS29 remain undigested.

Detection by *Acc*I digestion

To further verify whether mutation at residue 198 conferred MBC-resistance, a 329 bp fragment (based on all of the sequence alignment) containing the codon of residue 198 was amplified from *tub*2 by the primers PF1 5'-GCATGATGGCCACCTTCTC-3', which recognized the DNA from 924 to 942, and PR1 5'-GAGCGAAGCCGACCATGAAG-3', which recognized the DNA from 1231 to 1253. The mutation at residue 198 created a *Acc*I restriction site (CGCG: from 1030 to 1033 in *tub*2), where the codes altered from GACGAG in wild-type strains to GACGCG in MBC-resistant strains. In that amplified fragments, there was no identical restriction site, and *Acc*I digestion yielded two DNA fragments of 107 and 222 bp in resistant strains, while the fragments from wild-type strains remained undigested. These products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide when exposure to ultraviolet light (Figure 2).

DISCUSSION

Resistant mutants of almost all fungi to carbendazim are closely associated with the single base-pair mutation, and results in the mutation of amino acid as well as the structure of fungicide-binding point in ß-tubulin. The amino acid mutation of residue 198 in ß-tubulin gene has been identified in MBC-resistant fungi such as *Neurospora* spp. (Fujimura et al., 1990; Koenradt et al., 1993), *Botrytis cinerea* (Yarden and Katan, 1993), *Venturia inaequelis* (Koenradt et al., 1992), *Aspergillus nidulans* (Jung et al., 1992). Besides, different mutant points in other fungi such as residue 200 in *Neurospora* spp. (Koenradt et al., 1993), *B. cinerea* (Yarden and Katan, 1993) and *V. inaequalis* (Koenraadt et al., 1992), residue 241 in *Saccharomyces cerevisiae* (Thomas et al., 1985), residue 50 in *Fusarium moniliforme* (Yank and Dickman, 1996) were also identified and reported.

In this study, we discovered amino acid mutant points in residues 181,198, 237 and 363 according to the alignment of *tub*2 between resistant and wild-type strains of *C.gloeosporioides* in Mango. We can deduce that the mechanism of MBC-resistant in *C.gloeosporioides* was the same as that of other fungi. However, in order to determine whether these amino acid mutations confer MBC-resistance, they must be detected individually. According to single base mutation, allele-specific PCR and enzyme assays were employed. The results showed that only the amino acid mutation at residue 198 was closely correlated with MBC-resistant. residues 181, 237 and 363 did not have the same correlation.

Anthracnose is an important disease of Mango in tropical regions in China. Very frequently, MBC-resistant strains were detected in Hainan and Guangdong province in recent years as a result of the use of benzimidazole frequently and singly over a long period of time. The ß-tubulin gene was fully cloned and sequenced and we have outlined the mechanism of MBC-resistance in *C. gloeosporioides* of Mango. On the basic of above results, we can quickly detect the resistant group and its developments in the field by the allele-specific PCR assay. It is significant to the control of Mango anthracnose and the rational use of fungicides. Besides, in genetic engineering research, the high carbendazim-resistant gene can be used as a dominant selectable marker in filamentous fungal transformation experiments similar to *bar* (Charles et al., 1987) and *hph* (Gento et al., 2003) genes, as well as benomyl-resistant gene cloned from *Aspergillus flavus* (Seip et al., 1990) and *Neurospora crassa* (Marc et al., 1986).

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