MUTATION AT CODON 198 OF *TUB2* GENE FOR CARBENDAZIM RESISTANCE IN *COLLETOTRICHUM GLOEOSPORIOIDES* CAUSING MANGO ANTHRACNOSE IN THAILAND

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Received: January 1, 2011
Accepted: July 4, 2001

Abstract: Screening of field isolates of *Colletotrichum gloeosporioides* from various mango cultivars from markets and orchards in Thailand identified 113 carbendazim-resistant isolates. Isolates with a highly-resistant phenotype (HR) grew well on Potato Dextrose Agar (PDA) amended with carbendazim even at ≥500 mg/l. Isolates with carbendazim-resistant phenotype had a conspicuous mutation at a particular site in the β-tubulin (*TUB2*) gene sequence. The sequence of *TUB2* in HR isolates showed a single nucleotide transversion of adenine to cytosine, resulting in a substitution at codon 198 from glutamic acid (GAG) in wild type to alanine (GCG) in HR isolates. This is the first report of the molecular determination of field isolates for benzimidazole fungicide resistance in Southeast Asia in *C. gloeosporioides* causing mango anthracnose.

Key words: fungicide resistance, point mutation, tree fruits

INTRODUCTION

Anthracnose disease caused by *Colletotrichum gloeosporioides* is one of the most important field and postharvest diseases of mangoes from Thailand (Akem 2006). The pathogen causes leaf spot and blight, blossom blight, wither tip, twig blight, fruit rot, and tree dieback in mangoes. Fungicides of the benzimidazole group (*e.g.*, benomyl, carbendazim, and thiabendazole) have been widely used to control this pathogen (Prior *et al*. 1992; Ploetz 2003; Prakash 2004; Akem 2006) and these chemicals effectively suppress and control a wide variety of mango diseases. However, the efficacy of these chemicals has declined over time. The reason for the decline is most likely due to the appearance and development of fungicide-resistant isolates, which have been observed in many other regions of world (Staub 1991; Brent and Hollomon 1998; Ma and Michailides 2005; Ishii 2006; Deising *et al*. 2008).

Carbendazim is one of the broad-spectrum benzimidazole fungicides with systemic activity (Davides 1986). Carbendazim is recommended for control of anthracnose disease of many crops (Prakash 2004; Duamkhanmanee 2008). This fungicide is known as a specific inhibitor of microtubule assembly by binding to the β-subunit of β-tubulin and interfering with microtubule formation during mitosis of cell division (Davides 1986; Steffens *et al*. 1996; Ma and Michailides 2005). However, overuse for a long period may select mutant isolates on the target site of the chemical in β-tubulin and increase the population of fungicide-resistant isolates, causing a major problem for farmers (Staub 1991; Brent and Hollomon 1998; Ma and Michailides 2005; Deising *et al*. 2008). Deising *et al*. (2008) reported that two years of intensive use of benzimidazoles induced development resistance in the apple scab fungus *Venturia inaequalis* and polyphageous grey mold fungus *Botrytis cinerea* in the field. The appearance of acquired fungicide resistance in fields has become an important factor in limiting the fungicide efficacy and useful lifetime of important disease control strategies in Thailand. The cost is also increased because farmers are forced to increase the dosage and frequency of application of substitutable chemicals.

Phenotypic responses of benzimidazole-resistant pathogens have been reported in Thailand (Farungsang and Farungsang 1992; Farungsang *et al*. 1994), India (Kumar *et al*. 2007), South Africa (Sander *et al*. 2000), Korea (Yoon *et al*. 2008), Malaysia (Sariah 1989), and England (Taggart 1999). The resistance of *C. gloeosporioides* at both phenotypic and genetic mutation levels has also been reported in leguminous weeds in the United States (Buhr and Dickman 1994), various fruit crops in Japan (Chung *et al*. 2006), pepper and strawberry in Korea (Kim *et al*. 2007), the herbaceous ornamental perennial genus *Limonium* in Israel (Maymon *et al*. 2006), citrus in the United States and Brazil (Peres *et al*. 2004), and mango in China

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(Ru-Lin and Jun-Sheng 2007). In most cases, benzimidazole resistance is correlated with point mutations of particular nucleotides in the β-tubulin genes, especially the *TUB2* gene (Buhr and Dickman 1994). The mutation of a single nucleotide in the sequence of the *TUB2* gene, results in a reduction of the binding affinity of the fungicide to β-tubulin due to altered amino acid sequences at the benzimidazole-binding site (Davides 1986; Deising *et al*. 2008). The occurrence of amino acid substitutions has been observed at certain codons with the major target at 198 proved by sequence identification of field isolates as well as site-directed mutagenesis (Freeman *et al*. 2000; Ma and Michailides 2005).

Appearances of fungicide-resistant isolates in Southeast Asia have also been shown in Thailand (Farungsang and Farungsang 1992; Farungsang *et al*. 1994) and Malaysia (Sariah 1989), but the mechanism of the resistance has not been examined yet. The objectives of this research were to evaluate the mechanism of carbendazim-resistance in various field isolates of *C. gloeosporioides* obtained from mango. Evaluation was meant to be done by determining the benzimidazole sensitivity and sequencing of the partial region of the *TUB2* gene expected to be the major target site responsible for benzimidazole resistance.

MATERIALS AND METHODS

Collection and identification of field isolates

Fruits and leaves naturally infected by the pathogen were collected from various mango cultivars from markets and orchards of Thailand, in 2007–2008. The causal agents were isolated by a basic tissue transplanting technique. Tissues pieces were placed on Potato Dextrose Agar (PDA) plates and incubated at 30°C. The plates were observed daily until mycelium grew, and each isolate was subcultured to new PDA and grown at 30°C for 7–10 days. A conidial suspension of 0.5 ml was spread with a sterile glass rod on the surface of a WA Petri dish. Conidial germination was observed under a light compound microscope. A piece of agar containing a single germinated conidia was removed and transferred to the new PDA Petri dish.

The morphological characteristics of colony color and conidia size and shape of all isolates incubated on PDA at 30°C for 7 days, were examined for identification by referring to Sutton (Sutton 1992). Additional information for taxonomic determination was obtained by polymeraze chain reaction (PCR) amplification and sequence comparison of the Internal Transcribed Spacer (ITS) of the rDNA region. Approximately 100 mg of mycelia from each isolate were ground to a fine power in liquid nitrogen with a mortar and pestle. Genomic DNA was extracted using a NucleoSpin® kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Extracted DNA was used as the template for PCR amplification with a species-specific primer CgInt (5′-GGC CTC CCG CCT CCG GGC GG-3′) designed from the *ITS1* region of *C. gloeosporioides* and the conserved primer ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al*. 1990; Mills *et al*. 1992; Freeman *et al*. 2000). PCR reactions were performed in a total volume of 50 µl containing 10 to 100 ng of genomic DNA, 5 µl of 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs (iNtRON Biotechnology, Seoul, Korea), 50 pmol each primer, and 1 unit of *Taq* polymerase (Fermentas, Vilnius, Lithuania). All PCR reactions were carried out in a PTC-100™ programmable thermal controller (MJ Research, Waltham, MA, USA) with a hold of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. The PCR product was separated by electrophoresis on 1% agarose gels (Research Organics, Cleveland, OH, USA) with a 100-bp sharp DNA marker (RBC Bioscience, Taipei, Taiwan) as a size standard. PCR products purified by ethanol precipitation were direct-sequenced by the dideoxy chain termination method using an ABI-Prism Dry Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), and an automated fluorescent DNA sequencer (Model 310, Applied Biosystems) following the manufacturer's instructions. Sequence similarity and alignment analyses were performed using the Basic Local Alignment Search Tool (BLAST) in the GenBank or National Center for Biotechnology Information (NCBI) database.

Screening of fungicide-resistant isolates

The resistance of 150 randomly selected isolates of *C. gloeosporioides* to carbendazim was screened using mycelial growth assays. A 5 mm in diameter mycelial plug of each isolate was cut from the margins of colonies and transferred onto PDA amended with carbendazim at the concentration of 0.1, 1, 10, 100, 500 (field-use recommended concentration), or 1,000 mg/l. Carbendazim was added to PDA after autoclaving. The plates were inoculated and incubated at 30°C, followed by an evaluation of the diameter of each colony. The resistance to carbendazim was evaluated and grouped into one of four representative reaction phenotypes: highly resistant (HR) which means able to grow on carbendazim at ≥ 500 mg/l, moderately resistant (MR) to £100 mg/l, weakly resistant (WR) to £10 mg/l and sensitive (S) to £1 mg/l, as previously described (Farungsang and Farungsang 1992; Peres *et al*. 2004).

Analysis of carbendazim resistance using partial sequence of β-tubulin gene

DNA extracted from each isolate as described above was used as the template for PCR with a set of species-specific primers TB2L (5′-GTT TCC AGA TCA CCC ACT CC-3′) and TB2R (5′-TGA GCT CAG GAA CAC TGA CG-3′) designed from the sequence of the β-tubulin gene of *C. gloeosporioides* (Buhr and Dickman 1994). PCR reactions were performed in a total volume of 50 µl, containing 10 to 100 ng of genomic DNA, 5 µl of 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs (iNtRON Biotechnology), 50 pmol of each primer, and 1 unit of *Taq* polymerase (Fermentas). All PCR reactions were carried out in a PTC-100™ programmable thermal controller (MJ Research), with a hold of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 35°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. The PCR product was separated by electrophoresis on 1% agarose gels (Research Organics) with a 100-bp sharp DNA marker (RBC Bioscience) as

the size standard. Nested PCR amplification using a second set of PCR primers CTB2F1 (5′-TCC AAG ATC CGT GAG G-3′) and CTB2R (5′-AAG AAG TGG ACG GG-3′) was performed in a total volume of 50 µl reaction mixture containing 36 µl of dH_2O, 1 µl of templates (10X dilution of first PCR product), 5 µl of 10X PCR buffer, 5 µl of dNTPs, 1 µl of each primer, and 1 µl of *Taq* polymerase. The second round of PCR mixture was incubated at 95°C for 5 min, followed by 40 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. The second PCR product was separated by electrophoresis on 1% low melting point gel and subcloned into a pGEM-T^{Ò}-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Sequences of the product were obtained as described above. Sequence similarity and alignment analyses were performed using BLAST in the GenBank or NCBI database with the implemented ClustalX (Thompson *et al*. 1997).

RESULTS

Species identification

Pathogen were isolated from anthracnose symptomatic mangoes of various cultivars including Chaokhunthip, Chok Anon, Farlun, Kaew, Khiaomorakod, Khiaosawoey, Lin Nguhao, Mahacharnok, Mankhunsi, Namdokmai, Naree Luemrang, Okrong, Phebanlat, Phimsen, Raet, Salaya and Talapnak. The color of aerial mycelia from most isolates was grayish white to dark grey. They formed hyaline, cylindrical conidia with a size of 4.2–5.1 x 15.4–20.6 µm. These morphological characteristics were consistent with those of *C. gloeosporioides* reported by Sutton (1992). Several isolates from each cultivar were randomly selected for further identification using a molecular technique. A partial genomic region about 450 bp of ITS rDNA from each isolate was amplified by PCR using the primer set of CgInt and ITS4. Comparisons of sequences from these isolates showed that sequences from all isolates tested had the highest similarity with *C. gloeosporioides*.

Carbendazim sensitivity assays

Among the isolates of *C. gloeosporioides*, 150 isolates were randomly selected and tested for carbendazim resistance by growth assays on PDA amended with carbendazim at concentrations of 0.1–1,000 mg/l. The isolates consisted of 113 isolates (75.3%) with the HR phenotype containing 18 isolates (12%) obtained from leaves and 95 isolates (63.3%) from fruits, respectively. There were 37 isolates (24.7%) of the S phenotype consisting of 28 isolates (18.7%) from leaves and nine isolates (6%) from fruits (Fig. 1). None of the isolates showed WR or MR phenotype.

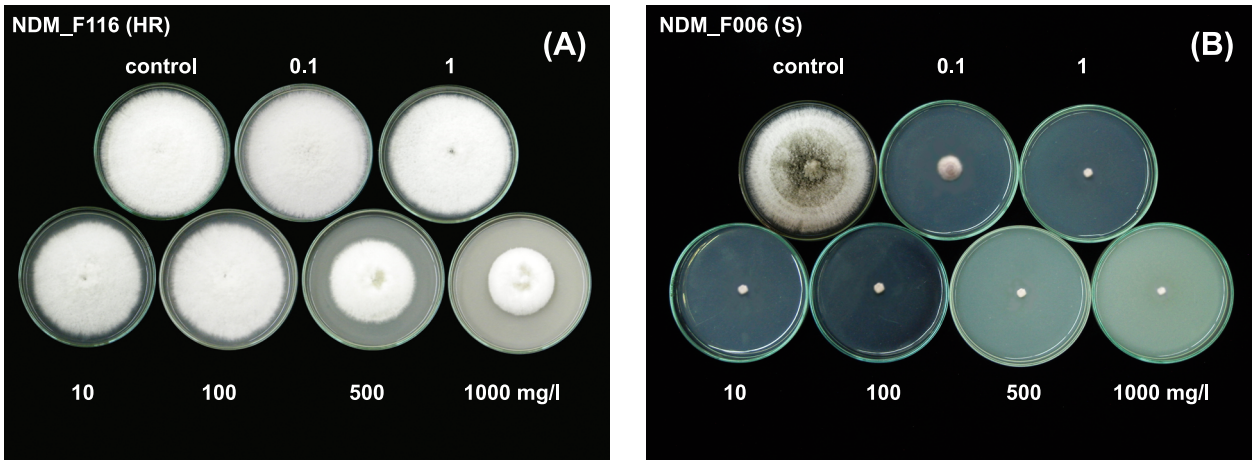

Fig. 1. Carbendazim-resistance assays of *C. gloeosporioides* causing mango anthracnose on potato dextrose agar supplemented with carbendazim at the control (0), 0.1, 1, 10, 100, 500, and 1,000 mg/l
(A) – highly resistant; (HR) – phenotype; (B) – sensitive; (S) – phenotype

Analysis of *TUB2* gene fragments

A partial region (341 bp in length) of the *TUB2* gene sequence from 30 randomly selected isolates of the HR phenotype and 27 isolates of the S phenotype from various cultivars of mango was amplified. Then, the sequences were compared with the same region of the sequence of wild-type *C. gloeosporioides* f. sp. *aeschynomene* (accession no. U14138). Alignment and comparison of the sequences identified a nucleotide substitution, at position 1,286 from adenine (A) to cytosine (C) in the *TUB2* gene sequence that resulted in a substitution of glutamic acid (G<u>A</u>G) in the wild type and alanine (G<u>C</u>G) in the HR isolates (Table 1).

Table 1. Correlation between phenotypes of carbendazim resistance and a point mutation at codon 198 in the *TUB2* gene of *C. gloeosporioides* isolates causing anthracnose disease in various mango cultivars in Thailand

Mango cultivar	Isolation code	Phenotype[a]	Nucleotide and amino acid sequence in codon position
			195 196 197 **198** 199 200 201 202 203 204
	U14138[b]	wild type	AAC TCC GAC **GAG** ACC TTC TGC ATT GAC AAC N S D **E** T F C I D N
1	2	3	4
Chaokhunthip	CKT_L044	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Chok Anon	CAN_F125	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	CAN_L080	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	CAN_L105	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	CAN_F095	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	CAN_F146	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Farlun	FL_F003	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	FL_L079	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	FL_F066	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Kaew	K_L120	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	K_F103	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Khiaomorakod	KMK_L088	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	KMK_F135	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	KMK_L058	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Khiaosawoey	KSW_L062	S	AAT TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	KSW_L085	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Lin Nguhao	LNG_L031	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
Mahacharnok	MCN_L059	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	MCN_L070	S	AAT TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	MCN_L121	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	MCN_L056	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Mankhunsi	MKS_L086	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N

1	2	3	4
Namdokmai	NDM_F006	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_F118	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_L057	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_L067	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_L068	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_L071	S	AAT TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_L096	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NDM_F002	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F012	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F014	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F018	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F026	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F027	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F038	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F061	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F106	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F110	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F116	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_F130	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	NDM_L078	HR	AAT TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Naree Luemrang	NLR_L048	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	NLR_L047	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Okrong	OR_L040	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	OR_F126	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Phebanlat	PBL_F102	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	PBL_F033	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	PBL_F076	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	PBL_F131	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N

1	2	3	4
Phimsen	PS_L032	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	PS_F114	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
	PS_L082	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N
Raet	R_L087	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
Salaya	SLY_L017	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
Talapnak	TLN_L060	S	AAC TCC GAC GAG ACC TTC TGC ATT GAC AAC N S D E T F C I D N
	TLN_L065	HR	AAC TCC GAC GCG ACC TTC TGC ATT GAC AAC N S D A T F C I D N

[a]responses to carbendazim on PDA; [b]accession number; HR – highly resistant; S – sensitive

DISCUSSION

Carbendazim, an inhibitor of tubulin biosynthesis, effectively controls the pathogen of mango anthracnose, *C. gloeosporioides* (Davides 1986; Steffens *et al*. 1996; Ma and Michailides 2005). Carbendazim is also one of the most popular fungicides used to control many fruit diseases in Thailand. About 75% of randomly selected isolates of *C. gloeosporioides* from various cultivars of mango in markets and orchards in Thailand, showed the HR response to carbendazim. As many other examples indicate, repeated application of the chemical enhances development of resistant isolates against carbendazim (Sariah 1989; Farungsang and Farungsang 1992; Farungsang *et al*. 1994; Taggart *et al*. 1999; Sanders *et al*. 2000; Kuo 2001; Kumar *et al*. 2007; Yoon *at al*. 2008). Highly resistant isolates of *C. gloeosporioides* likely developed under conditions of repeated carbendazim application in the fields. There has been a recent significant increase in fungicide use in Thailand (Thapinta and Hudak 2000). The appearance of such fungicide-resistant isolates severely limits the fungicide efficacy and lifetime of disease control strategies for farmers.

Multiple studies indicate that the basis of benzimidazole resistance is closely associated with single nucleotide mutations in the β-tubulin genes that change the structure of the fungicide-binding point (Orbach *et al*. 1986; Fujimura *et al*. 1992; Koenraadt *et al*. 1992; Yarden and Katan 1993; Buhr and Dickman 1994; Yan and Dickman 1996; Gafur *et al*. 1998; Albertini *et al*. 1999; Peres *et al*. 2004; Chung *et al*. 2006; Davidson *et al*. 2006; Ziogas *et al*. 2009). The major target for amino acid substitution in the *Colletotrichum* species against benzimidazoles, including carbendazim, is at codon 198 in the *TUB2* gene. The mutations cause amino acid substitutions at this codon of glutamic acid to glycine, lysine, alanine, or valine (Koenraadt *et al*. 1992; Buhr and Dickman 1994; Yan and Dickman 1996; Peres *et al*. 2004; Sholberg *et al*. 2005; Chung *et al*. 2006; Ru-Lin and Jun-Sheng 2007). Our partial sequence analyses of the *TUB2* genes in Thailand field isolates of *C. gloeosporioides* also showed a nucleotide mutation occurring at codon 198, an adenine (A) to cytosine (C) substitution. This resulted in an amino acid substitution of glutamic acid (GAG) in the S to alanine (GCG) in the HR isolates. The presence of the mutation was always correlated with the HR phenotype of the isolates tested.

Analyses of other examples of benzimidazole resistance in field isolates of *Colletotrichum* species including mango anthracnose in China (Ru-Lin and Jun-Sheng 2007), postbloom fruit drop disease of citrus in the United States and Brazil (Peres *et al*. 2004), anthracnose disease of various fruit crops in Japan (Chung *et al*. 2006), anthracnose disease of *Limonium* spp. in Israel (Maymon *et al*. 2006), anthracnose of pepper and strawberry in Korea (Kim *et al*. 2007) and the United States (Buhr and Dickman 1994), gave the same mutation at the specific site. The amino acid mutation at codon 198 in the β-tubulin gene has been also identified in other fungi such as *Botrytis cinerea* (Yarden anf Katan 1993; Ziogas *et al*. 2009), *Cercospora beticola* (Davidson *et al*. 2006), *Monilinia fructicola* (Koenraadt *et al*. 1992; Ma *et al*. 2003), *Mycosphaerella fijiensis* (Cañas-Gutiérrez *et al*. 2006), *Neurospora crassa* (Fujimura *et al*. 1992), *Penicillium* spp. (Baraldi *et al*. 2003; Koenraadt *et al*. 1992; Sholberg *et al*. 2005), *Sclerotinia homoeocarpa* (Koenraadt *et al*. 1992), *Tapesia yallundae, T. acuformis* (Albertini *et al*. 1999), *Venturia inaeqalis*, and *V. pirina* (Koenraadt *et al*. 1992). In all the cases, the mutation induces resistance to benzimidazole. Different mutation points in other codons such as at codon 6 in *M. fructicola* (Ma *et al*. 2003), codon 50 in *Fusarium moniliforme* (Yan and Dickman 1996), codon 167 in *Cochliobolus heterostrophus* (Gafur *et al*. 1998), *P. expansum* (Baraldi *et al*. 2003), and *N. crassa* (Orbach *et al*. 1986), codon 200 in *B. cinerea* (Yarden and Katan 1993), *C. gloeosporioides* (Chung *et al*. 2006), *P. italicum*, *P. aurantiogriseum*, *V. inaeqalis*, *V. pirina* (Koenraadt *et al*. 1992), *T. yallundae* and *T. acuformis*, and codon 240 in *T. yallundae* and *T. acuformis* (Albertini *et al*. 1999) were also found, but these mutations result in different levels of resistance (weak or moderate) to the fungicide. In these cases, amino acid substitutions caused by site-direct changes at particular target codons were demonstrated to be the cause of fungicide resistance by loss or reduction of the binding affinity to benzimidazole associated with the amino acid changes in β-tubulin (Davides 1986; Steffens *et al*. 1996; Ma and Michailides 2005). In this study, we examined the

putative target site of point mutation at codon 198 of the *TUB2* gene in field isolates of *C. gloeosporioides* causing mango anthracnose in Thailand because many isolates showed high resistance to carbendazim. The fungicide resistance might result from mutations at other sites as well. This is the first report of a typical mutation associated with carbendazim resistance in Southeast Asia. It is an important warning, calling for careful management of fungicide applications used with the goal of achieving effective control. Once HR isolates occupy a field, as is likely the case in this study, reduction of the population of these isolates is very difficult (Ishii 2006). Thus, a rotation of different fungicide groups (Staub 1991; Buhr and Dickman 1994) or Integrated Pest Management (IPM) (Yenjit *et al*. 2004; Prabakar *et al*. 2008; Singh *et al*. 2008) for control of this disease is an immediate solution to prevent the spread of HR isolates caused by application of a single type of fungicide in this region.

In conclusion, the sequence of *TUB2* in the isolates with an HR phenotype showed a single nucleotide transversion of adenine to cytosine, resulting in a substitution at codon 198, which encodes glutamic acid (GAG) in the wild type and converts it to alanine (GCG) in HR isolates.

ACKNOWLEDGEMENTS

We thank Dr. H. Kunoh, Dr. S. Itoh, and Dr. H. Ishii for their valuable advice; Ms. C. Miyake for technical assistance. This research is partially supported by the Thailand Research Fund and the Center for Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education, Thailand.

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