CHAPTER 4

Detection of Carbendazim-resistant *Colletotrichum* spp. Using the Second Beta-Tubulin Gene Sequences

4.1 Introduction

The appearance of fungicide resistance to fungal pathogens is a key factor in limiting the efficacy and useful of fungicides, which results in increasing higher production costs. The effective plant disease control is necessary to determine the resistibility of the pathogen to fungicides. Thus, fungicide resistance becomes important to understand the mechanism of action at the molecular level in a particular group of chemical fungicides. Carbendazim is a member of benzimidazoles; a group of wide range data is available on genetic features involving in fungicide resistance to determine by one, or a few genes which result in changes to the tubulin protein (Fujimura *et al.*, 1992; Ma and Michailides, 2005; Deising *et al*., 2008). Resistance to benzimidazoles in *Aspergillus* spp., *Neurospora* spp., *Venturia* spp. and *Colletotrichum* spp. is derived from mutations to the gene encoding beta-tubulin (Ma and Michailides, 2005). Fungicide-resistant isolates in Southeast Asia have appeared in Thailand (Farangsang and Farungsang, 1992; Farungsang *et al.*, 1994) and Malaysia (Sariah, 1989).

The resistance of *Colletotrichum* spp. to benzimidazole fungicide, at a phenotypic and genetic mutation level, has been reported subsequently in

leguminous weeds in the USA (Buhr and Dickman, 1994), 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 fruit in the USA and Brazil (Peres *et al*., 2004), and mango in China (Ru-Lin and Jun-Sheng, 2007). Recently, benzimidazole resistance was reported to correlate with nucleotide point mutations in the beta-tubulin (*TUB*) gene, especially the second beta-tubulin (*TUB2*) gene as it has a high degree of homology and expresses in ungerminated conidia, conidiophores, and vegetative mycelia (Buhr and Dickman, 1994). The mutation of a single nucleotide in the sequence of the *TUB2* gene resulted in reduction of the binding affinity of the fungicide to beta-tubulin, due to alter amino acid sequences at the benzimidazole-binding site (Davidse, 1986; Deising *et al*., 2008). The occurrence of amino acid substitutions has been observed at some codons such as 6, 50, 167, 198, 200 or 240, as reviewed by Ma and Michailides (2005). The major target of 198 was proven by sequence identification of the target sites in field isolates (Fujimura *et al.*, 1992; Ma and Michailides, 2005). Regarding these codon mutations, site-directed mutagenesis supports the mutation hypothesis of benzimidazole fungicide group, depending on the field of the substituting amino acid, and conferring with widely different levels of resistance.

The objectives of this chapter were as follows:-

1. To analyze point mutation in sequencing the partial region of the *TUB2* gene, which is expected to be the major target site responsible for carbendazim resistance, and correlated with phenotypes of *C. gloeosporioides* isolates that differ in their resistance to carbendazim.

4.2 Materials and Methods

Genetic analysis of carbendazim resistance using a partial sequence of the beta-tubulin gene

DNA extraction

All isolates from the *Colletotrichum* species identified in Chapter 3, using the partial sequence of ITS regions, were genomic DNA for analysis. Each isolate was grown on PDA at RT (28-30^{O}C) for 14 days. Approximately 100 mg of mycelia of each isolate was 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. The determination of DNA concentration and electrophoresis were followed in Chapter 3.

Amplification of the second beta-tubulin region

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 *TUB2* region of *C. gloeosporioides* (Brent and Hollomon, 1998; Buhr and Dickman, 1994) (Figure 4.1). 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 (iNtRON Biotechnology), 25 mM of $MgCl_2$ (iNtRON Biotechnology), 10 mM of 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^{TM} programmable thermal controller (MJ Research), with a hold of 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 35°C (T_m), and 72°C, and a final

extension for 5 min at 72°C. The PCR product was separated using electrophoresis on 1% agarose gel (Research Organics) with a 100 bp DNA marker (RBC Bioscience) as a standard size. Nested PCR amplification, using a set of 2nd PCR primers CTB2F1 (5'-TCC AAG ATC CGT GAG G-3') and CTB2R (5'-AAG AAG TGG ACG GG-3') (Figure 4.1), was performed in a total volume of 50 µl of the reaction mixture, containing 36 µl of dH_2O, 1 µl of templates (first PCR product with ten times dilution), 5 µl of 10X PCR buffer, 5 µl of dNTPs, 1 µl of each primer, and 1 µl of *Taq* polymerase. The second PCR mixture was incubated at 95°C for 5 min and denatured through 40 cycles of 1 min at 95°C, at 50°C (T_m), and at 72°C, and a final extension for 5 min at 72°C.

Cloning and sequencing

The second PCR product was separated by electrophoresis on 1% low melting point gel and subcloned into pGEM-T®-T Easy Vector Systems (Promega, WI, USA) using the target band of the second PCR product, following the manufacturer's instruction. The sequences of the product were obtained from both strands, using the dideoxy chain termination method with an ABI Prism Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) and automated fluorescent DNA sequencer (Model 310, Applied Biosystems). The sequencing was performed in the Plant Pathology Laboratory, Faculty of Agriculture, Kagawa University, Japan.

Sequence similarity and alignment analyses were performed using BLAST in GenBank or the NCBI database with the implemented CLUSTAL W and BioEdit program.

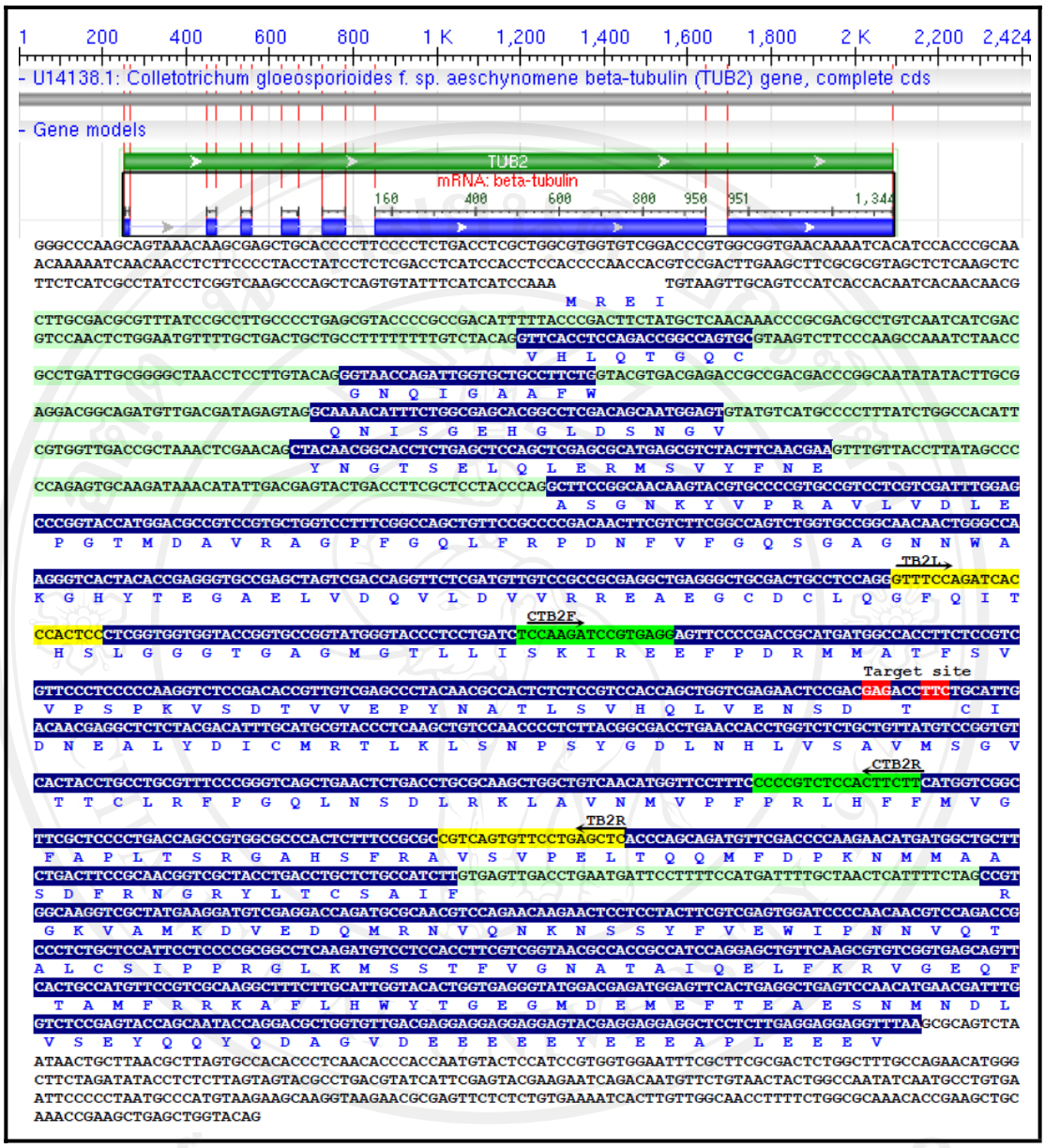

Figure 4.1 Primers in nested polymerase chain reaction (PCR); TB2L (5' GTT TCC AGA TCA CCC ACT CC 3') and TB2R (5' TGA GCT CAG GAA CAC TGA CG 3'), CTB2F1 (5' TCC AAG ATC CGT GAG G 3') and CTB2R (5' AAG AAG TGG ACG GG 3') synthesized in the partial region of the second beta-tubulin gene based on the nucleotide sequence of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (accession no. U14138).

4.3 Results

Genetic analysis of the second beta-tubulin gene fragments.

Thirty isolates of the Car^{HR} group, 27 of Car^{S} group and one of the Car^{MR} groups were randomly selected from various isolates of mango cultivars for identification through molecular techniques. The DNA of each isolate was amplified when the first PCR reaction was performed using TB2L and TB2R primers. The corresponding amplified PCR region was the *TUB2* sequence, and the first PCR product region was approximately 474 bp for all samples (Figure 4.2a). The first PCR product of each isolate was amplified when the second PCR reaction was performed using CTB2F1 and CTB2R primers. The second PCR product region was approximately 341 bp (Figure 4.2b).

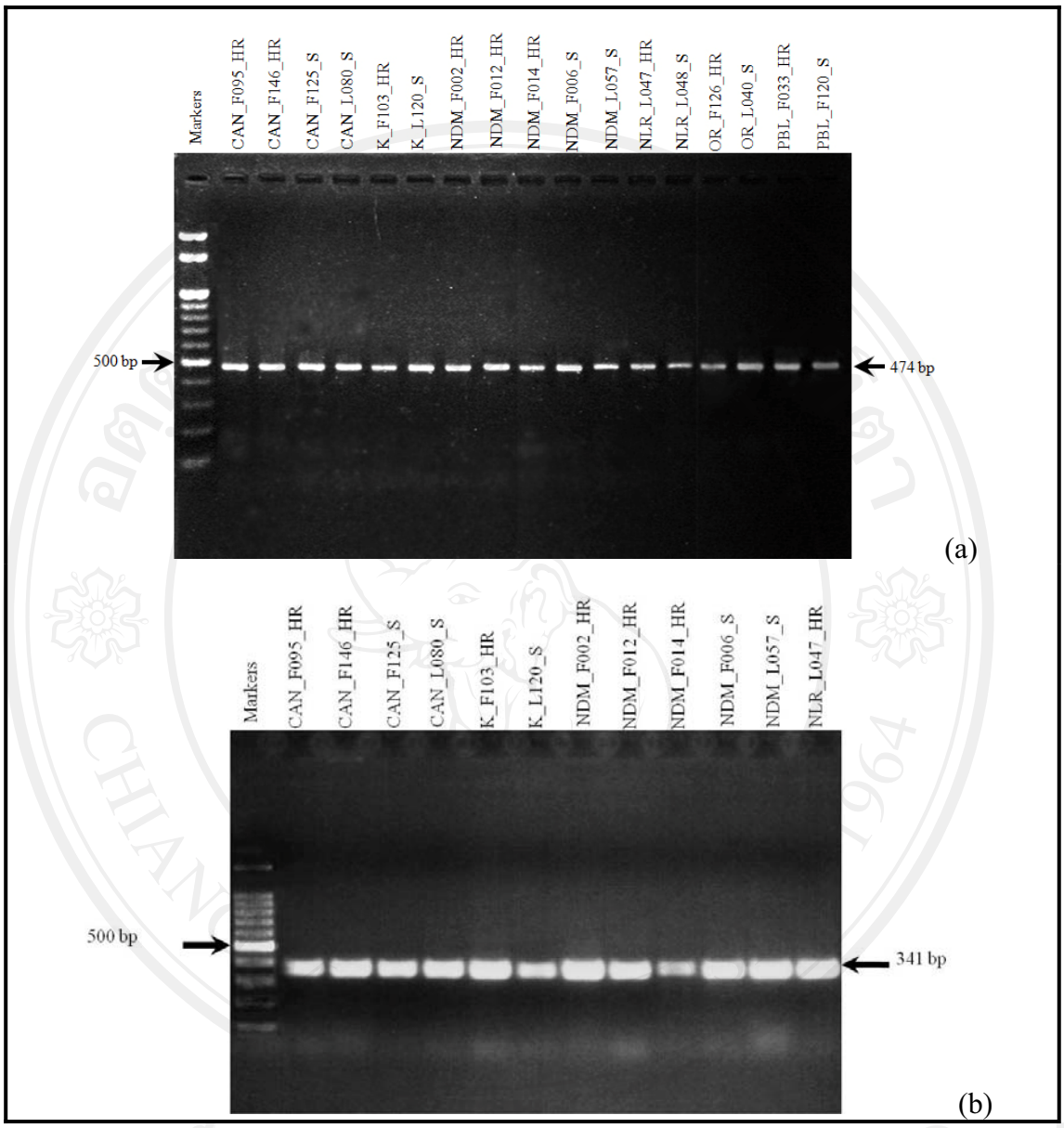

Figure 4.2 PCR amplification of the partial region of the second beta-tubulin gene from various isolates of *Colletotrichum gloeosporioides* that cause mango anthracnose; primary PCR products using TB2L and TB2R primers (a) and the second PCR products using CTB2F and CTB2R primers (b). Markers are the 100 bp Ladder, and the arrow on the left side indicates the position of 500 bp in both (a) and (b) panels. Arrows on the right side mark the group-specific band in both panels.

The *TUB2* sequences retrieved from GenBank, including wild-type *C. gloeosporioides* f. sp. *aeschynomene* and the same isolate of *C. gloeosporioides* from ITS sequences referenced in the phylogenetic analyses in Chapter 3 [isolate code L11 (benomyl-resistant) and P1 (benomyl-sensitive)], were included in this study for comparison. The wild type sequences were published in Applied and Environmental Microbiology (accession no. U14138). The *TUB2* sequences of L11 (benomyl-resistant) and P1 (benomyl-sensitive) isolates had accession number DQ084509 and DQ084514, respectively (Table 4.1).

Each isolate, high-quality sequences of 341 bp in length, was used for analysis (Appendix 2). The nucleotide sequences details were compared with the same sequence region of wild-type *C. gloeosporioides* f. sp. *aeschynomene*, benomyl-resistant and –sensitive *C. gloeosporioides*.

Table 4.1 The second beta-tubulin sequences used in genetic analyses from GenBank

Pathogen	Isolate code	Host	Location	Accession no.	References
C. gloeosporioides f. sp. *aeschynomene*	wild-type	Northern jointvetch	the USA	U14138	Buhr and Dickman (1994)
C. gloeosporioides	L11 (Benomyl-resistant)	Statice	Israel	DQ084509	Maymon *et al*. (2006)
	P1 (Benomyl-sensitive)			DQ084514	

Alignment and comparison of the sequences identified several nucleotide substitutions and a mutation at position 1,286 in the *TUB2* gene sequence. This resulted in alteration of the deduced amino acid at codon 198 and an adenine (A) to cytosine (C) transversion which were found in all isolates of the Car^{HR} phenotype tested. This mutation resulted in the substitution of glutamic acid (G<u>A</u>G) in the wild type, Car^{MR} and Car^{S} to alanine (G<u>C</u>G) in the Car^{HR} phenotype, and was associated with the phenotype conferring with carbendazim-resistance. Futhermore, a mutation at position 1,292, resulting in the alteration of the deduced amino acid at codon 200 and thymine (T) to adenine (A) transversion, was found only the Car^{MR} phenotype tested isolates. This mutation resulted in the substitution of phenylalanine (T<u>T</u>C) in the wild type, Car^{HR} and Car^{S} to tyrosine (T<u>A</u>C) in the Car^{MR} phenotype, and associated with the phenotype conferring to carbendazim-resistance (Figure 4.3, 4.4 and Table 4.2).

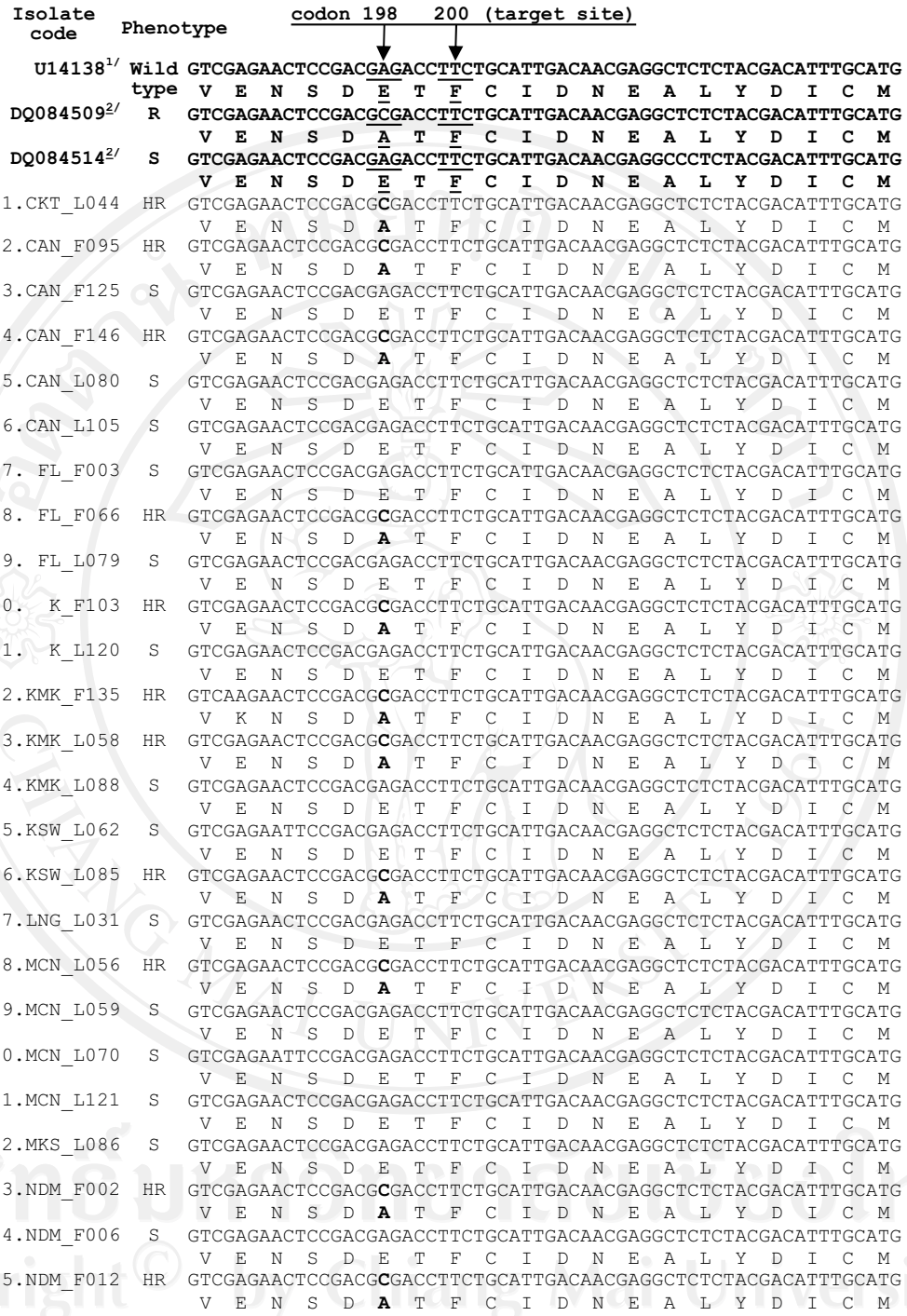

Figure 4.3 Comparison between deduced nucleotide and amino acid sequences of the second beta-tubulin from *Colletotrichum gloeosporioides* f. sp. *aeschynomene* ([1/]Buhr and Dickman, 1994), benomyl-resistant and –sensitive *C. gloeosporioides* ([2/]Maymon *et al*., 2006) at the target sites of benzimidazole (Peres *et al*., 2004.) and the carbendazim-resistant phenotype of *C. gloeosporioide*s isolates causing anthracnose disease in various mango cultivars.

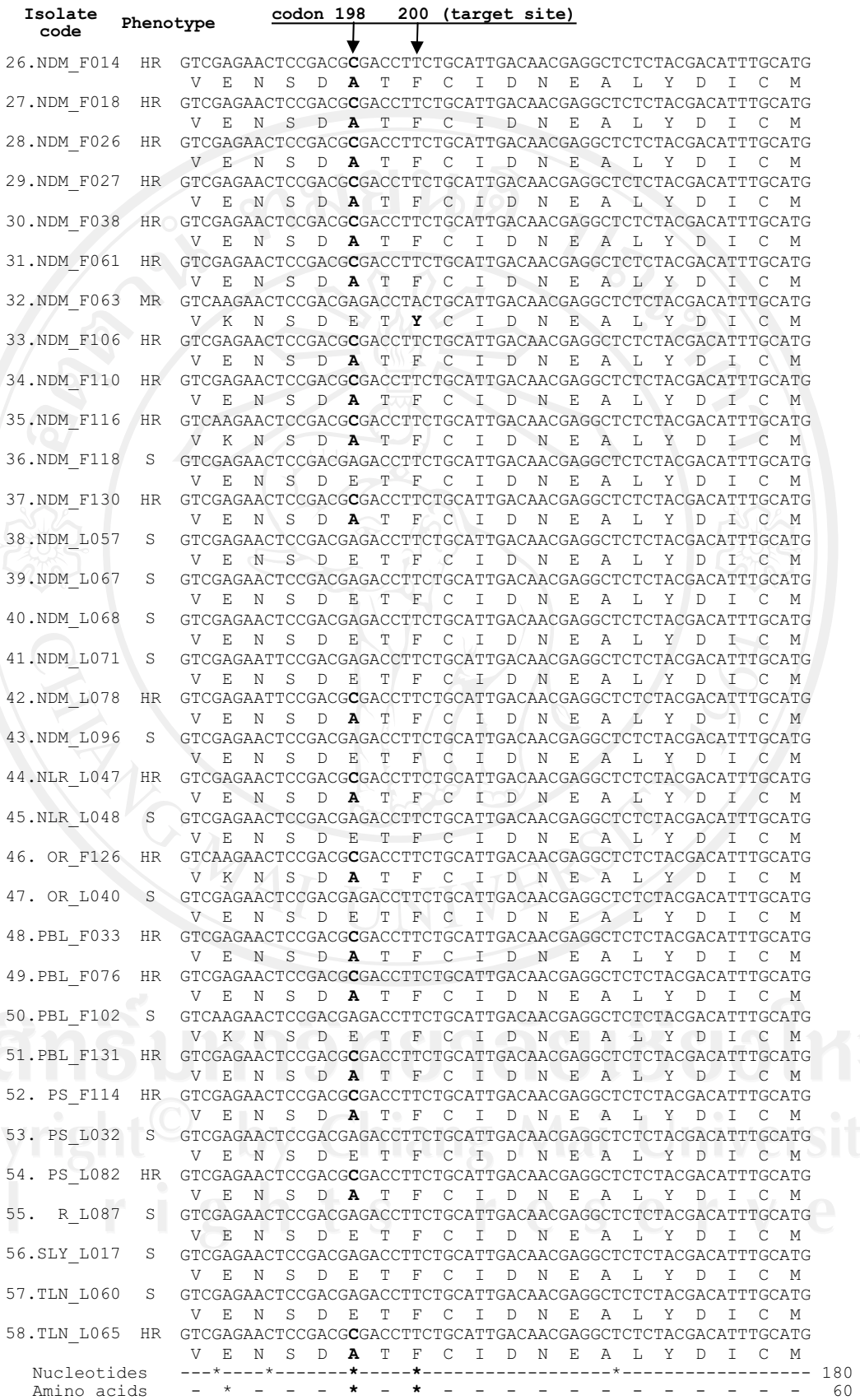

Figure 4.3 Continued.

Table 4.2 The nucleotide and amino acid substitution in the second beta-tubulin gene at codon 198 and 200 causing changes to the carbendazim resistibility

Phenotype	Genotype			
	Codon 198		Codon 200	
	Nucleotide	Amino acid	Nucleotide	Amino acid
U14138[1/] (Wild type)	(G**A**G) Adenine	Glu (E) Glutamic acid	(T**T**C) Thymine	Phe (F) Phenylalanine
DQ084509[2/] (Benomyl-resistant)	(G**C**G) Cytosine	**Ala (A) Alanine**	(T**T**C) Thymine	Phe (F) Phenylalanine
DQ084514[2/] (Benomyl-sensitive)	(G**A**G) Adenine	Glu (E) Glutamic acid	(T**T**C) Thymine	Phe (F) Phenylalanine
Highly resistant (HR) (30 isolates)	(G**C**G) Cytosine	**Ala (A) Alanine**	(T**T**C) Thymine	Phe (F) Phenylalanine
Sensitive (S) (27 isolates)	(G**A**G) Adenine	Glu (E) Glutamic acid	(T**T**C) Thymine	Phe (F) Phenylalanine
Moderately resistant (MR) (1 isolate)	(G**A**G) Adenine	Glu (E) Glutamic acid	(T**A**C) Adenine	**Tyr (Y) Tyrosine**

[1/]Accession no. of wild type *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Buhr and Dickman, 1994).
[2/]Accession no. of benomyl-resistant and –sensitive *C. gloeosporioides* (Maymon *et al*., 2006)

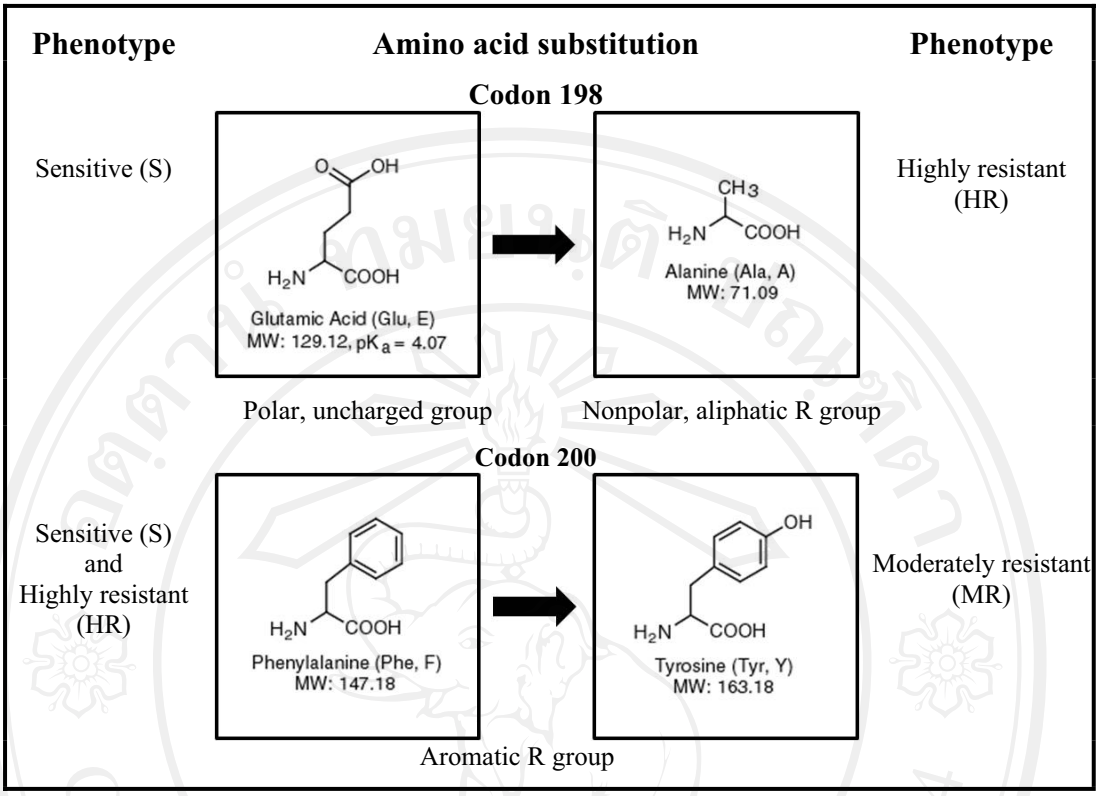

Figure 4.4 Amino acid substitution in the second beta-tubulin at codon 198 and 200 can change the carbendazim resistibility.

4.4 Discussion

Multiple studies indicated that the basis of resistance of almost all fungi to benzimidazol is closely associated with a single nucleotide mutation in the beta-tubulin genes that changes 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 against benzimidazoles, for amino acid substitution in the *Colletotrichum* species, includes carbendazim at codon 198 in the *TUB2* gene, and this mutation causes an amino acid substitution at the 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). This research finding on partial sequence analyses of the *TUB2* genes in Thai isolates of *C. gloeosporioides* showed a nucleotide mutation occurring at codon 198 and an adenine (A) to cytosine (C) substitution the resulted in the amino acid substitution of glutamic acid (G<u>A</u>G), in the Car^{S} phenotype, with alanine (G<u>C</u>G) in the Car^{HR} phenotype. The presence finding of the mutation was correlated with the Car^{HR} phenotype of all tested isolates which agreement with previous reports on 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). In addition, amino acid mutation at codon 198 in the beta-tubulin gene has been also identified in other fungi such as *Botrytis cinerea* (Yarden and Katan, 1993; Ziogas *et al*., 2009), *Cercospora beticola* (Davidson *et al*., 2006), *Monilinia fructicola* (Koenraadt *et al*., 1992; Ma *et al*., 2003), *Mycosphaerella fijiensis* (Cańas-Gutiérrez *et al*., 2006), *Neurospora crassa* (Fujimura *et al*., 1992), *Penicillium* spp. (Koenraadt *et al*., 1992; Baraldi *et al*., 2003; Sholberg *et al*., 2005), *Sclerotinia homoeocarpa* (Koenraadt *et al*., 1992), *Tapesia yallundae, Tapesia acuformis* (Albertini *et al*., 1999), *Venturia inaeqalis*, and *Venturia pirina* (Koenraadt *et al*., 1992).

In this case, the mutation induces resistance to benzimidazole. However, there was the occurrence of the only isolate of Car^{MR} level which showed codon 200 substitution in agreement with previous reports on *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*, (Albertini *et al*., 1999).

However, 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), and codon 240 in *T. yallundae* and *T. acuformis* (Albertini *et al*., 1999) were also found, but these mutations resulted 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, through loss or reduction of the binding affinity to benzimidazole associated with the amino acid changes in beta-tubulin (Davidse, 1986; Steffens *et al*., 1996; Ma and Michailides, 2005). The result in this research found that different mutation point between codon 198 in highly resistant and codon 200 in moderately resistant showed different carbendazim-resistant levels.

Result in this study confirmed that the putative target site of point mutation at codon 198, of the *TUB2* gene in field isolates of *C. gloeosporioides* causing mango anthracnose for the first time in Thailand. All isolates responsed with a high level of resistance to carbendazim. Although this resistance might result from a mutation at another site, or even multiple gene mutations. It is suggested that a typical mutation

associated with carbendazim resistance in Thailand is an important warning for the careful management of fungicide applications to achieve effective control. Once if the isolates with the Car^{HR} phenotype occupy a field, it would be possible to reduce the pathogen population as also reported by Ishii (2006).

This research provides the information of fungicide resistance on the incidence and establishment of *C. gloeosporioides* in mangoes in Thailand. In next chapter, alternative control measure was investigated by testing various types of chitosan to control mango anthracnose to solve the problem of resistant build up.

4.5 Conclusion

The sequence of *TUB2* in the isolates with Car^{HR} phenotype showed a single nucleotide transversion of adenine (A) to cytosine (C), resulting in a substitution at codon 198, which encodes glutamic acid (GAG) in the wild type and converts it to alanine (GCG) in these isolates. Moreover, Car^{MR} phenotype showed a single nucleotide transversion of thymine (T) to adenine (A), resulting in a substitution at codon 200, which encodes phenylalanine (TTC) in the wild type and converts it to tyrosine (TAC) in these isolates.