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Traits Improvement by Ion-beam and Gamma-ray Irradiation in Entomopathogenic Fungi

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Traits Improvement by Ion-beam and Gamma-ray Irradiation in Entomopathogenic Fungi

(イオンビームおよびガンマ線を用いた 昆虫病原糸状菌の改良)

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ABSTRACT

The entomopathogenic fungi such as *Isaria fumosorosea* Wize, *Beauveria bassiana* (Bals.-Criv.) Vuill., and *Metarhizium anisopliae* (Met.) Sorokin, are important agents for the biological control of insect pests, however these fungi are highly susceptible to fungicides and incompatible to high temperatures. This research was aimed to develop fungicide tolerant or resistant mutants of *I. fumosorosea* and *B. bassiana*, and thermotolerant mutants of *M. anisopliae* by mutageneses using ion beams and/or gamma rays and thereby improve the potential for application of these fungi as microbial control agents in IPM programs of insect pests. Six benomyl-tolerance mutant isolates of *I. fumosorosea* (Ib-34 (200 Gy) and Ib-421 (300 Gy) from ion-beam irradiation, Gr-5 and Gr-22 (both 1000 Gy) from gamma-ray irradiation, and GrIb-8 and GrIb-9, both from dual irradiation by 500 Gy of gamma rays followed by 200 Gy of ion beams) and two benomyl-resistant mutant of *B. bassiana* (BB22 and BB24, both from ion beams at 150 Gy) were obtained in this study. Five mutant isolates with more tolerance to heat stress were also obtained derived from two wild-types of *M. anisopliae* i.e: AcMa5-gr-1 and AcMa5-gr-2 (100 Gy) from gamma-ray irradiation, AcMa5-gr-3 (1000 Gy) from gamma-ray irradiation, AcMa5-ib (300 Gy) from ion-beam irradiation and PaMa02-ib (100 Gy) from ion-beam irradiation. In an EC_{50} assay of mycelial growth, five of six mutant isolates of *I. fumosorosea* were more than 2000-fold those observed for the wild-type isolate (EC_{50}: > 5000 mg l^{-1} c.f. EC_{50}: 2.5 mg l^{-1} for the wild-type isolate) meanwhile, BB22 and BB24 were >500-fold more tolerant to benomyl compared with the wild-type isolate. In an EC_{50} assay of conidial germination, two mutant isolates of *I. fumosorosea* 2-fold more tolerance than the wild-type isolate,

however, there was no significant difference in conidial germination of *B. bassiana* between the wild-type and the mutant isolates. Mycelial growth of both mutant isolates of *B. bassiana* also enhanced tolerance to thiophanate-methyl, but reduced tolerance to diethofencarb. And for *I. fumosorosea* the mutant isolates showed enhanced tolerance to other fungicides at recommended field application rates. No differences were observed at the *β-tubulin* gene between the wild-type and the mutant isolates of *I. fumosorosea*, suggesting that the enhanced benomyl-tolerance was not attributable to mutations in that gene. A mutation was found at position 198 of the *β-tubulin* gene in the mutant isolates of *B. bassiana*, with a substitution of glutamate for alanine (E198A). Thermotolerant mutants of *M. anisopliae* increased upper thermal limit (38–39 ^{o}C) for vegetative growth by 2–3 ^{o}C compared to that of the wild-type isolates. All mutants grew fastest at 25^{o}C as same as the wild-type isolates. At 1×10^{7} conidia ml^{-1}, four mutants derived from a wild-type isolate caused high mortalities of 86–100% in rice weevil adults, which were not significantly different from a mortality caused by the wild-type isolate. However, one mutant derived from another wild-type isolate almost lost virulence. All mutants revealed no mutation in the neutral trehalase gene (*Ntl*) which possibly relates to thermotolerance and also no mutation in *β-tubulin* gene and *ifT1* which relates to fungicide tolerance. Ion-beam and gamma-ray irradiations may be useful tools for improving characteristics such as increasing fungicide tolerance and thermotolerance in the entomopathogenic fungi. However, the resulting mutants should be carefully evaluated for unpredictable negative effects. This is the first report for enhanced thermotolerance induced by ion-beam and gamma-ray irradiations in entomopathogenic fungi.

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CHAPTER 1

GENERAL INTRODUCTION

Pesticides have been widely used and make significant contribution in maintaining food. However, their usages do not always decrease yield losses. For example, in the US, even 10-fold usage of insecticide from 1944 to 1989, total yield losses caused by pest insects increased nearly double from 7 to 13% (Pimentel et al., 1992). Many negative effects of pesticides have also been reported, i.e. crop pollination problem and honeybee losses, crop and crop product losses, ground water and surface water contamination, fish wildlife, microorganism lost, including beneficial natural enemies and pesticide resistance (Pimental et al., 1992). Until now, pest resistance caused by pesticides still becomes problems in insect pests management in the world (Cloyd, 2010).

Many methods have been performed to reduce resistance caused by pesticides such as pesticide rotation and mixtures, however, these two methods are not fully effective (Cloyd, 2010). Combination of all the management methods including alternative pest management strategies such as cultural, sanitation and biological control which is known as Integrated Pest Management (IPM), have been reported as the effective method for reducing resistance (Cloyd, 2010). Biological control method with microbial insecticides has been widely used and take an important part in IPM that can be applied coincide by the other management methods (Shah and Pell, 2003; Khan et al., 2012a). Many kinds of the microbial insecticide, including entomopathogenic fungi have been commercially produced and widely used for pest insect management in glasshouses and

field worldwide (de Faria and Wraight, 2007; Copping, 2009).

In plant disease management, fungicides are commonly used as the main treatment for reducing damage of the cultivated plant caused by fungal plant pathogens. However, fungicide applications will directly cause negative effects on the entomopathogenic fungi applied for pest insects (Clark et al., 1982; Loria et al., 1983; Saito and Yabuta, 1996; Pell et al., 2010; D'Alessandro et al., 2011). The harmful effects of fungicides on entomopathogenic fungi are typically avoided by employing extended intervals between applications of each agent (Gardner et al., 1984; Bruck, 2009), these intervals can complicate the effective and practical use of both products in the field. Thus, we need to develop entomopathogenic fungi which are highly tolerant to fungicides which can be applied coincide with fungicides for plant disease management.

On the other hand, entomopathogenic fungi are also seriously affected by abiotic factors, i.e. UV, humidity, and temperature (Castrillo et al., 2005; Zimmermann, 2007a, b, 2008; Jaronski, 2010). Among those abiotic limiting-factors, temperature is one of the most important key factors for using entomopathogenic fungi. In the high temperature condition, such as more than 35^{o}C, it will seriously affect conidial germination and persistence, vegetative growth, sporulation, and/or infection of entomopathogenic fungi (Arthurs and Thomas, 2001; Cabanillas and Jones, 2009; Darbro et al., 2011; Dimbi et al., 2004; Fargues et al., 1997; Inglis et al., 1997; Li and Feng, 2009; Thomas and Jenkins, 1997; Vidal et al., 1997; Davidson et al., 2003), which may reduce their efficacy especially in hot seasons, tropical and subtropical regions, or glasshouses with elevated temperatures. To solve this problem, we also need to develop entomopathogenic fungi tolerant to heat stress.

One way to overcome incompatibility to fungicides and high temperatures has been to develop fungicide-tolerant and thermotolerant mutants of entomopathogenic fungi by selection on chemically amended media (Shapiro-Ilan et al., 2002, 2011; Butters et al., 2003), by transformation (Bernier et al., 1989; Valadares-Inglis and Inglis, 1997; Pfeifer and Khachatourians, 1992; Inglis et al., 1999), by UV radiation (Kim et al., 2005; Rangel et al., 2006), or by exposure to mutagenic agents such as $NaNO_2$ (Zou et al., 2006; Song et al., 2011).

To date, ion-beam and gamma-ray irradiations are mostly used for mutant development in breeding of crop and microorganism (Zengliang, 2006; Anuntalabhocai et al., 2011; Piri et al., 2011). Recently ion beams have been identified as a potential tool for mutagenesis in micro-organisms because of their characteristic point-like mutations, higher mutation frequency and broader mutation spectrum (Matuo et al., 2006; Shikazono et al., 2003, 2005; Tanaka et al., 2010; Toyoshima et al., 2012). For ion beams, exposure of *Cordyceps militaris* (L.) Link to ion beams successfully generated mutant isolates capable of enhanced production of cordycepin, a medical substance (Das et al., 2008, 2010). However, there are little reports in the literature on the use of ion-beam irradiation to improve traits for biological control agents of entomopathogenic fungi. Gamma rays have also been reported as a successful mutagenic agent in entomopathogenic fungi (Paccola-Meirelles and Azevedo, 1991; Kava-Cordeiro et al., 1995), although there are no reports describing enhanced fungicide resistance.

Entomopathogenic fungi, *Isaria fumosorosea* Wize, *Beauveria bassiana* (Bals. -Criv.) Vuill., and *Metarhizium anisopliae* (Met.) Sorokin, are now commercially available as microbial insecticides and have been widely used as biological control agents (Shah and Pell, 2003; de Faria and Wright, 2007). This research was aimed to

develop fungicide tolerant and resistant mutants of *I. fumosorosea* and *B. bassiana*, respectively, and thermotolerant mutants of *M. anisopliae* by mutageneses using ion beams and/or gamma rays and thereby improve the potential for application of these fungi as microbial control agents in IPM programs of insect pests.

CHAPTER 2

ENHANCED FUNGICIDE-TOLERANCE IN *ISARIA FUMOSOROSEA*

2.1. Introduction

Isaria fumosorosea has been used as a biological control agent for many kinds of insect pests belonging to order Coleoptera, Diptera, Hemiptera, Lepidoptera, Thysanoptera, etc. (Zimmermann, 2008). In ornamentals, this fungus is useful to control pest insects such as Thrips (Thysanoptera) (Cloyd, 2009; Arthurs et al., 2013) and leafminers (Wekesa et al., 2011). However, the fungus is incompatible to fungicides used in IPM programs of pest insects (D'Alessandro et al., 2011).

Benomyl, ethyl 1-[(butylamino)carbonyl]-1H- benzimidazol-2-ylcarbamate, is one of broad spectrum systemic fungicides which is in benzimidazole fungicides and widely used for controlling various plant diseases (Edgington et al., 1971; Erwin, 1973). However, applications of benomyl negatively affect to survival of entomopathogenic fungi. For example, benomyl inhibits mycelial growth and/or conidial germination in *B. bassiana* (Sabbour et al., 2011; Khan et al., 2012b), *M. anisopliae* (Yáñez and France, 2010; Khan et al., 2012b), and *Entomophtora planchoniana* (Lagnaoui and Radcliffe, 1998). Because of its toxicity, broad spectrum and wide usage, this fungicide was used as a model for increasing tolerance to fungicide in *I. fumosorosea* (Chapter 2) and *B. bassiana* (Chapter 3).

2.2. Materials and Methods

2.2.1. Fungal preparation

A wild-type isolate (PF-3110) of *I. fumosorosea* that had been isolated from the sweet potato whitefly, *Bemisia tabaci* B biotype in 1990 was cultured in Petri dishes (90 mm diameter) containing Sabouraud's dextrose agar (SDA; Difco, BD Biosciences, NJ) at 23 ± 1°C for 3 weeks in darkness. Conidial suspensions were prepared by scraping the conidia/mycelia into sterile 0.1% Tween 80 and then filtering the mixture through sterile cloth (0.2 mm mesh size) to provide a suspension of conidia. Before experimentation, the germination rate of conidia was determined on SDA at 23 ± 1°C (> 95%).

2.2.2. Ion-beam and gamma-ray irradiation

Conidia of the wild-type isolate were irradiated with carbon-ion beams ($^{12}C^{5+}$, 121.8 keVμm^{-1}) accelerated by an azimuthally varying field cyclotron at the Takasaki Ion Accelerators for Advanced Radiation Application site (Gunma, Japan) and/or gamma rays (^{60}Co, 0.2 keV μm^{-1}) at the Food Irradiation Facility, Japan Atomic Energy Agency (Gunma, Japan), respectively.

2.2.3. Fungicides against which mutants were evaluated

Eight commercial fungicides were used: benomyl [50% wettable powder (WP),

Sumitomo Chemical, Japan], thiophanate-methyl (70% WP, Nippon Soda, Japan), iprodione (50% WP, Nippon Soda, Japan), diethofencarb (25% WP, Sumitomo Chemical, Japan), chlorothalonil (40% WP, Kumiai Chemical Industry, Japan), polycarbamate (75% WP, Dow Chemical Japan, Japan), myclobutanil [25% emulsifiable concentrate (EC), Dow Chemical Japan, Japan] and triflumizole (15% EC, Ishihara Sangyo Kaisha, Japan). Each fungicide was added to autoclaved SDA once it had cooled to below 50^{o}C and mixed using a stirrer before pouring the agar and allowing it to set. The eight fungicides used in this experiment belong to different classes of chemical, except benomyl and thiophanate-methyl, which are both benzimidazoles.

2.2.4. Relationship between irradiation dose and survival rates

The relationship between irradiation dose and conidial survival rate was determined as follows. For ion-beam irradiation, 50 µl of the conidial suspension (2.5×10^3 conidia ml^{-1}) was spread on 20 ml SDA in each replicate plastic Petri dish (60 mm diameter). The dishes were covered with a polyimide film (Kapton 30EN, Du Pont-To-ray, Japan) and irradiated at a range of doses (0, 50, 100, 200, 300, 400, 500 and 600 Gy). For gamma-ray irradiation, 100 µl of the conidial suspension (2.0×10^3 conidia ml^{-1}) was used in each Petri dish (90 mm diameter), the dishes were covered with plastic lids, and the range of doses tested was 0, 30, 100, 300, 1000 and 3000 Gy. All dishes were incubated at 23 ± 1^{o}C in darkness, and five replicates were made for each dose. Survival rates were determined based on the number of colonies that grew on SDA after 3 days and used to select appropriate doses for the production of mutants.

2.2.5. Production of mutants with potential tolerance to benomyl

Conidia from 3 ml of a suspension (1.0×10^8 conidia ml^{-1}) of the wild-type isolate of *I. fumosorosea* were transferred to each of three replicate 47-mm-diameter cellulose membrane filters with a pore size of 0.45 µm (Millipore, Merck Millipore, Germany) using filtration equipment (Swinnex, Merck Millipore, Germany). The filters were individually placed in plastic Petri dishes (60 mm diameter for ion-beam irradiation and 50 mm diameter for gamma-ray irradiation). Prior to ion-beam irradiation, the Petri dish lids were replaced with polyimide film. Conidia collected on the membrane filters were irradiated either with ion beams or with gamma rays at a range of doses selected from the relationship between irradiation dose and survival rate determined in the previous experiment (n = 3 per dose). Each treated filter was then transferred to a vial containing 3 ml of sterile Sabouraud's dextrose broth (SDB) and agitated with a sterile glass rod to detach the conidia from the filters. The resulting conidial suspensions were incubated at 20 ± 1°C in darkness overnight to remove unstable mutations through cell division (germination). From each suspension, 200 µl was spread on to SDA into which 1000 mg l^{-1} benomyl had been incorporated, in each of five/six Petri dishes (90 mm diameter). The dishes were then incubated at 23 ± 1°C in darkness for 7 days. Well-grown colonies were assumed to be benomyl-tolerance mutants and isolated onto fresh SDA for further evaluation; colonies that differed in shape and colour from the wild-type isolate were excluded. In addition, the effectiveness of dual irradiation with both gamma rays and ion beams for inducing mutations was examined in the wild-type isolate; irradiation doses of 0, 50 or 500 Gy of gamma rays were followed by 0, 100 or 200 Gy of ion beams, respectively. After the conidia on the membrane filters were irradiated with

gamma rays, they were placed in Petri dishes containing SDA at 23 ± 1°C and incubated in darkness overnight to remove unstable mutations through cell division (germination). This was followed by ion-beam irradiation and subsequent treatment procedures were as described previously for single ion-beam irradiation.

2.2.6. Evaluation of levels of resistance to benomyl in mutants

2.2.6.1. Mycelial growth

The EC_{50} (effective concentration of benomyl to reduce mycelial growth by 50%) values were determined for wild-type and mutant isolates based on mycelial growth on SDA that contained different quantities of benomyl. Mycelial plugs (4 mm diameter) of each isolate were excised from the margins of colonies growing on SDA. The plugs were then placed individually into the centre of Petri dishes (90 mm diameter) containing 30 ml SDA to which benomyl had been added (0, 0.1, 0.3, 1, 3, 10 or 30 mg l^{-1} for the wild-type isolate and 0, 300, 1000, 3000 or 5000 mg l^{-1} for the mutant isolates; n = 3 per dose). Dishes were incubated at 25 ± 1°C in darkness for 7 days and mean colony diameter was determined from two perpendicular measurements of each colony, excluding 4 mm to account for the diameter of the original inoculation plug. Each colony diameter was then divided by the mean colony diameter obtained for the same isolate when grown on SDA containing no fungicide, to provide relative growth rates. The EC_{50} value for each isolate was estimated using the growth rates obtained for each dose by linear regression using the software package SPSS Statistics (SPSS, 2009).

2.2.6.2. Conidial germination

Three 20 µl aliquots of conidial suspension (1.0×10^6 conidia ml^{-1}) from each of the mutant isolates and the wild-type isolate were placed individually onto 30 ml of SDA containing benomyl (10, 25, 50, 100, 250, 500 or 1000 mg l^{-1}) in 90 mm diameter Petri dishes. In addition aliquots were placed onto control plates that had been produced in the same way but without fungicide. Each aliquot was covered with a sterile glass coverslip (18 mm × 18 mm) and there were five replicate dishes (including controls and each containing three aliquots) per dose for each isolate. The dishes were incubated at 25 ± 1°C in darkness for 16 h. After the addition of lactophenol cotton blue solution, the percentage germination of approximately 100 conidia per aliquot was determined under a microscope (Axio Imager 2, Zeiss, Germany). The mean percentage germination for each replicate dish was arcsine square-root transformed, and the EC_{50} of each isolate was estimated as above, using data from seven of the doses (10, 25, 50, 100, 250, 500 or 1000 mg l^{-1}); the data from other doses were not used for EC_{50} estimation because they achieved germination rates of almost 0% or equivalent to the control (100%).

2.2.7. Tolerance of mutants to other fungicides

Growth of benomyl-tolerance mutants on SDA to which one of eight fungicides, including benomyl, had been incorporated at the recommended field application rate was evaluated. The methods were the same as described above, except that dishes were incubated for 8 days. Each isolate was also cultured in the absence of fungicides (control), and there were five replicate dishes per fungicide. The tolerance ratio (TR)

was obtained using the following formula: (mean colony diameter for fungicide/ mean colony diameter for control) mutant/(mean colony diameter for fungicide/mean colony diameter for control) wild type. For each mutant, the mean colony diameter for each fungicide was statistically compared to the mean colony diameter for the control using a Mann–Whitney's U-test (SPSS, 2009).

2.2.8. DNA sequencing of *β-tubulin* locus

After culturing the wild-type and mutant isolates on SDA at 20°C for 3 days, genomic DNA was extracted from the conidia of each isolate using a FastDNA Spin Kit (MP Biomedicals, UK). To isolate the partial *β-tubulin* locus, PCR was conducted using a PrimeSTAR GXL DNA polymerase (Takara Bio, Japan), the purified genomic DNA as a template and two specific primers, Isaria_betatub-R and Metarhizium_beta-tub-F2 (Table 1), under the following conditions: 1 cycle of 98°C for 20 s, 30 cycles of 98°C for 10 s, 68°C for 2 min, 1 cycle of 68°C for 5 min and then storage at 4°C. PCR was done in 50 µl reaction volumes consisting of 5 µl genomic DNA (5 ng ml^{-1}), 10 µl 5 × PrimeSTAR GXL Buffer, 4 µl dNTP mixture, 1 µl PrimeSTAR GXL DNA Polymerase (TaKaRa Bio, Japan), 0.3 µl of each primer (50 µM) (Sigma Genosys, Japan) and 29.4 µl sterile distilled water. The PCR products were purified using a MinElute PCR Purification Kit (Qiagen, the Netherlands). To identify the mutation sites in the *β-tubulin* locus, DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, CA) and six specific primers (Table 1) with an ABI PRISM 377 DNA Sequencer (Life Technologies).

2.2.9. Response of mutants to temperature

We measured colony diameters (as the methods those used to assess benomyl-tolerance levels in the previous experiments but without benomyl), of mutant isolates and the wild-type isolate after 7 days of growth in darkness on SDA in 90 mm diameter Petri dishes (30 ml per dish) at 15, 20, 25, 30, 33 or 35^{o}C. There were three replicate dishes for each isolate at each temperature. At each temperature, the mean colony diameter for each mutant was compared statistically to the mean colony diameter of the wild-type isolate using the Mann-Whitney *U* test (SPSS, 2009).

2.2.10. Pathogenicity of mutants

Pathogenicity of mutants and the wild-type isolate were compared against tobacco whitefly, *Bemisia tabacci* (Genn.) (Homoptera: Aleyrodidae) in laboratory. The whiteflies were collected from cucumber plants grown in a glasshouse of Shizuoka University and reared on potted cabbage in container (40 cm × 40 cm × 40 cm) at 25 ± 1^{o}C and 16L: 8D. For each isolates, three cabbage leaves containing 30 individual of 2nd instar whitefly larvae were cut by 8 cm × 8 cm and sprayed with conidial suspensions (1 × 10^{6}, 10^{7} and 10^{8} conidia ml^{-1}) of the mutants and the wild-type isolate using hand sprayer. Mortality of the 2nd instar whitefly was recorded daily for 7 days and all cadavers transferred to 60 mm diameter Petri dishes with wet filter paper. The dishes of cadavers were incubated at 25 ± 1^{o}C in darkness to promote fungal development and those developing mycosis recorded. The percentage of mortality caused by mycoses was arcsine square-root transformed and the data for mutant isolates was compared

statistically to the data for the wild-type isolate using the Mann-Whitney *U* test (SPSS, 2009).

2.3. Results

2.3.1. Relationship between irradiation dose and survival rates

Ion-beam irradiation with a dose of 50 Gy was associated with high survival rates (64.6%), 500 Gy with very low survival rates (0.3%), and 600 Gy killed all the conidia (Table 2). Gamma-ray irradiation with doses below 300 Gy only slightly affected survival rates (> 78.1%), 1000 Gy was associated with very low survival rates (0.5%), and 3000 Gy killed all the conidia (Table 3).

2.3.2. Production of mutants with potential tolerance to benomyl

Six benomyl-tolerance isolates were established from colonies that appeared normal (Figure 1); Ib-34 (200 Gy) and Ib-421 (300 Gy) from ion-beam irradiation, Gr-5 and Gr-22 (both 1000 Gy) from gamma-ray irradiation, and GrIb-8 and GrIb-9, both from dual irradiation by 500 Gy of gamma rays followed by 200 Gy of ion beams.

2.3.3. Evaluation of levels of tolerance and conidial germination to benomyl in mutants

The wild-type isolate had an EC_{50} value of benomyl tolerance 2.5 mg l^{-1}, while five

mutant isolates, Ib-34, Ib-421, Gr-5, Gr-22 and GrIb-8, had EC_{50} values > 5000 mg l^{-1} (> 2000-fold more tolerance than the wild-type isolate; Table 4). Another mutant isolate, GrIb-9, also had a high EC_{50} value of 1855 mg l^{-1} (742-fold more tolerance than the wild-type isolate; Table 4).

The wild-type isolate had an EC_{50} value of conidial germination 151.31 mg l^{-1}, while two mutant isolates, GrIb-8 and GrIb-9, had EC_{50} values 307.71 and 229.74 mg l^{-1}, respectively (2.03 and 1.52-fold more tolerance than the wild-type isolate; Table 5). Meanwhile, another mutant isolate, Ib-34, had EC_{50} value of 121.88 mg l^{-1} (0.81-fold more sensitive than the wild-type; Table 5).

2.3.4. Tolerance of mutants to other fungicides

The wild-type and mutant isolates produced significantly smaller colonies on most of the fungicides at a field application rate compared with the control ($P < 0.01$ or $P < 0.05$; Table 6). As expected, however, all the mutant isolates had large TR values (4.48–6.46) when grown on media containing benomyl. Similarly, the mutants also had comparatively large TR values (1.30–1.69) when grown on media containing thiophanate-methyl, which, like benomyl, is a benzimidazole fungicide. For the other fungicides, the mutant isolates generally had TR values around 1, which indicated they had similar tolerance levels to the wild-type isolate, except for iprodione (TR: 1.05–2.05) and myclobutanil (TR: 1.16–4.99). These findings suggest that the mutant isolates may have multiple mechanisms conferring tolerance to several fungicides including benzimidazoles.

2.3.5. Sequence of *β-tubulin* locus

The *β-tubulin* locus of the wild-type and six mutant isolates was amplified by PCR. Two of the mutant isolates, Ib-34 and Gr-5, produced very weak amplification signals and were excluded from further analysis (Figure 2). The *β-tubulin* sequence in *I. fumosorosea* is 1727 bp (Song et al., 2011), but only partial genomic DNA sequences of the *β-tubulin* locus (1424 bp at nucleotide position from 304 to 1727) were determined for the wild-type and four mutant isolates and the *β-tubulin* sequences in the four mutant isolates, Ib-421, Gr-22, GrIb-8 and GrIb-9, were identical to those of the wild-type isolate (Figure 3).

2.3.6. Response to temperature

Three mutants i.e. Gr-22, Ib-34 and GrIb-8 grew significantly more slowly than the wild-type isolate at 15, 20, 25 ^{o}C ($P < 0.05$), and stop growing at 33^{o}C. One mutant, Gr-5, also stop growing at 33^{o}C. The other two mutants i.e. GrIb-9 and Ib-421 were still growing at 33^{o}C, the same as the wild-type isolate. The growth of Gr-5, GrIb-9 and Ib-421 were not significantly different than the wild-type isolate at 15^{o}C, 20^{o}C, 25^{o}C for Gr-5 and Ib-421 and 33^{o}C for GrIb-9 and Ib-421. The growth was significantly more slowly than the wild-type isolate at 25^{o}C and 30^{o}C for GrIb-9 and at 30^{o}C for Gr-5 (Figure 4). None of the isolates, including the wild-type isolate, grew at 35^{o}C.

2.3.7. Pathogenicity of mutants

Mortality in 2nd instars larva of *B. tabacci* was started 4 days after inoculation for both the wild-type and mutant isolate treatments. At 1×10^7 and 1×10^8 conidial ml^{-1} concentration, caused 98–99.2% and 99.8–100% mortality, respectively, which was not significantly different from the 99.9% (1×10^7 conidial ml^{-1}) and 100% (1×10^8 conidial ml^{-1}) mortality caused by the wild-type isolate ($P > 0.05$). However, at 1×10^6 conidial ml^{-1}) concentration, two mutants i.e. Ib-34 and GrIb-8, caused only 63.4% and 51.1% mortality, which was significantly less than 97.1% mortality caused by the wild-type isolate ($P < 0.05$). Only one mutant, GrIb-9 caused 76.7% mortality which was not significantly different from the wild-type isolate (Table 7).

2.4. Discussion

In previous studies, the highest mutation frequency obtained by ion-beam irradiation was associated with survival rates ranging from 1 to 10% *Saccharomyces cerevisiae* (Matuo et al., 2006) and *Aspergillus oryzae* (Toyoshima et al., 2012). Consequently, we selected irradiation doses of 100, 200, 300, 400 and 500 Gy for ion-beam irradiation and 400, 600, 800 and 1000 Gy for gamma-ray irradiation when attempting to produce mutants. Preliminary study revealed that those mentioned irradiation dose gave survival rate more than 1% and less than 50%.

This considerably enhanced tolerance in the mutant isolates was greater than that of *I. fumosorosea* benzimidazole-tolerance isolates produced using other methods. For example, carbendazim-tolerant mutants generated using the mutagen $NaNO_2$ exhibited a

maximum EC_{50} value of only > 1000 mg l^{-1}, which was still 830-fold more tolerance than the wild-type isolate (Song et al., 2011). Furthermore, benomyl-tolerance transformants generated using a polyethylene glycol-mediated procedure had a minimum inhibitory concentration of only 20 mg l^{-1} compared with 7.5 mg l^{-1} for the wild-type isolate (Inglis et al., 1999). Enhanced tolerance in the developed mutants may be sufficient to avoid the negative effects resulting from benomyl application in the field (500 mg l^{-1} is the recommended application rate). This is the first study to demonstrate that ion-beam and gamma-ray irradiations are potentially useful tools for inducing mutations that enhance fungicide tolerance in entomopathogenic fungi. As the increase in tolerance obtained separately with either irradiation method alone was considerable, combined irradiation may be unnecessary. Ion-beam irradiation facilities are now available worldwide. This method creates different characteristics in entomopathogenic fungi, because it causes a high mutation frequency and a broad mutation spectrum and creates point mutations in genes (Matuo et al., 2006; Zengliang, 2006; Tanaka et al., 2010; Toyoshima et al., 2012).

Benzimidazole fungicides, such as benomyl, are negatively cross-resistant to N-phenylcarbamate fungicides, such as diethofencarb (Fujimura et al., 1992a; Ziogas and Girgis, 1993; Leroux et al., 1999). However, developed benomyl-tolerance isolates exhibited similar or little more sensitivity to diethofencarb (TR: 0.84–1.09) than the wild-type isolate. Regardless of this, all mutants should be screened for undesirable mutations that may have occurred alongside the desired mutation conferring benomyl tolerance. For example, it will be important to compare the virulence of mutant and wild-type isolates against target pests to ensure that any benefits associated with benomyl resistance are not counteracted by any loss in virulence; these studies are

currently under way in our laboratories.

Most of the molecular studies on benzimidazole-resistant phytopathogenic fungi have focused on the replacement of amino acids at codon 198 and/or 200 in the *β-tubulin* locus (Fujimura et al., 1992b; Koenraadt et al., 1992; Koenraadt and Jones, 1993; Yarden and Katan, 1993; Albertini et al., 1999; Davidson et al., 2006; Schmidt et al., 2006; Kongtragoul et al., 2011). In addition, a similar mutation at codon 198 has been reported in $NaNO_2$-induced benomyl-tolerance mutants of *B. bassiana* (Butters et al., 2003; Zou et al., 2006). However, none of these mutations in the *β-tubulin* locus were detected in mutant isolates, suggesting that other mechanisms may be responsible for the observed enhancement in benomyl-tolerance. Song et al. (2011) showed that although benomyl-tolerance *I. fumosorosea* mutants did not possess any mutations at the *β-tubulin* gene, mutations were observed in the promoter region of the ABC transporter gene (*ifT1*). It is thus possible that similar mutations may have occurred in the developed benomyl-tolerance mutant isolates. These findings indicate that different mechanisms are conferred to benomyl-tolerance between *B. bassiana* and *I. fumosorosea*. Future studies will examine the possibility of mutations in other genes including *ifT1* using the developed mutants.

Table 1. Primers used for *β-tubulin* locus PCR amplification and sequencing of *I. fumosorosea*.

Primer	Sequence (5′ to 3′)	Use
Isaria_beta-tub-R	TTACATGGGCTCCTCAGCCTCA	PCR and sequencing
Beauveria_beta-tub-R2	CTTCATGGCAACCTTACCAC	Sequencing
Beauveria_beta-tub-R3	GAACAACGTCGAGGACCTG	Sequencing
Metarhizium_beta-tub-F2	CCAAATTGGTGCTGCTTTCTGG	PCR and sequencing
Beauveria_beta-tub-F2	CAGGGTTTCCAGATCACCC	Sequencing
Beauveria_beta-tub-F3	GAGGACCAGATGCGTAATGTG	Sequencing

Table 2. Relationship between ion-beam irradiation doses and conidial survival rates in the wild-type isolate of *I. fumosorosea*.

Dose (Gy)	No. colonies per plate (mean ± SE)	Survival rate[a]
0 (control)	88.7 ± 4.3	100
50	57.3 ± 10.5	64.6
100	33.3 ± 2.4	37.5
200	12.0 ± 1.0	13.5
300	3.7 ± 0.3	4.2
400	1.7 ± 0.3	1.9
500	0.3 ± 0.3	0.3
600	0.3 ± 0.3	0.3
700	0	0

[a]Survival rate = (mean number of colonies in irradiation / mean number of control colonies) × 100.

Table 3. Relationship between gamma-ray irradiation doses and conidial survival rates in the wild-type isolate of *I. fumosorosea*.

Dose (Gy)	No. colonies per plate (mean ± SE)	Survival rate[a]
0 (control)	140.0 ± 2.0	100
30	124.0 ± 9.0	88.6
100	115.7 ± 16.8	82.6
300	109.3 ± 5.9	78.1
1000	0.7 ± 0.3	0.5
3000	0	0

[a]Survival rate = (mean number of colonies in irradiation / mean number of control colonies) × 100.

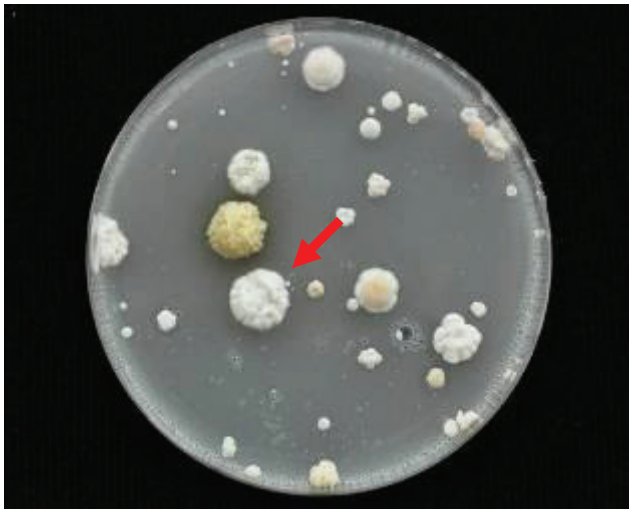

Figure 1. Benomyl-tolerant mutant colony derived from *I. fumosorosea* wild-type conidia irradiated with ion beams (400 Gy) plated on SDA to which benomyl had been added (1000 mg l^{-1}). Mutants should be indicated by arrows.

Table 4. Benomyl-tolerance in the wild-type and mutant isolates of *I. fumosorosea*.

Isolate	Linear regression				EC_{50} (mg l^{-1})	TR[a]
	Constant (95% CL)	Slope (95% CL)	*r*	*F*		
PF-3110 (wild-type)	1.508 (1.413 ~ 1.603)	–0.297 (–0.326 ~ –0.268)	0.984	482	2.5	
Ib-34	n.a.	n.a.	n.a.	n.a.	> 5000	> 2000
Ib-421	n.a.	n.a.	n.a.	n.a.	> 5000	> 2000
Gr-5	n.a.	n.a.	n.a.	n.a.	> 5000	> 2000
Gr-22	n.a.	n.a.	n.a.	n.a.	> 5000	> 2000
GrIb-8	n.a.	n.a.	n.a.	n.a.	> 5000	> 2000
GrIb-9	2.650 (2.448 ~ 2.853)	–0.343 (–0.375 ~ –0.310)	0.991	549	1855	742

[a]TR = EC_{50} value for the mutant isolate / EC_{50} value for the wild-type isolate.
n.a.: not analyzed.

Table 5. Benomyl-tolerance in conidial germination of the wild-type and mutant isolates of *I. fumosorosea*.

Isolate	Linear regression [2)]				EC_{50} (mg l^{-1})	TR [3)]
	Constant (95% confidential limit)	Slope (95% confidential limit)	*r*	*F*		
PF-3110 (wild-type)	–64.00 (–41.60 ~ –86.40)	189.50 (136.10 ~ 242.90)	0.863	38	151.31	
Ib-34	–35.26 (–18.85 ~ –51.68)	123.55 (84.42 ~ 162.68)	0.790	22	121.88	0.81
GrIb-8	–101.20 (–83.65 ~ –118.75)	301.80 (254.21 ~ 349.39)	0.961	155	307.71	2.03
GrIb-9	–57.61 (–37.84 ~ –77.37)	186.02 (138.90 ~ 233.14)	0.868	40	229.74	1.52

Table 6. Colony diameters (mm, mean ± SE) of the wild-type and mutant isolates of *I. fumosorosea* on SDA containing each fungicide at the recommended application rate and the TR[a] in parentheses.

Isolate	Benomyl (500 mg l^{-1})	Thiophanate-methyl (700 mg l^{-1})	Iprodione (500 mg l^{-1})	Diethofencarb (250 mg l^{-1})	Chlorothalonil (400 mg l^{-1})	Polycarbamate (937 mg l^{-1})	Myclobutanil (625 mg l^{-1})	Triflumizole (150 mg l^{-1})	No fungicide
Wild-type	3.4 ± 0.1** (1)	14.0 ± 0.2** (1)	6.2 ± 0.3** (1)	21.6 ± 1.2* (1)	16.3 ± 0.5** (1)	9.6 ± 0.5** (1)	2.0 ± 1.0** (1)	0.6 ± 0.1** (1)	25.4 ± 0.4
Ib-34	7.7 ± 0.3** (5.70)	9.4 ± 0.4 (1.69)	4.3 ± 0.2** (1.74)	7.2 ± 0.2** (0.84)	9.4 ± 0.2 (1.45)	5.6 ± 0.4** (1.47)	3.3 ± 0.2** (4.15)	0** (0)	10.1 ± 0.4
Ib-421	12.7 ± 0.9** (4.70)	14.5 ± 0.6** (1.30)	5.2 ± 0.2** (1.05)	18.7 ± 0.6 (1.09)	13.2 ± 1.0** (1.02)	9.1 ± 0.2** (1.19)	4.9 ± 1.2** (3.08)	0.1 ± 0.1** (0.17)	20.2 ± 0.1
Gr-5	17.9 ± 0.2** (5.31)	22.4 ± 0.2** (1.60)	9.2 ± 0.7** (1.50)	19.1 ± 0.2** (0.89)	16.7 ± 0.4** (1.03)	7.9 ± 0.4** (0.83)	2.3 ± 0.2** (1.16)	0.7 ± 0.2** (1.17)	25.2 ± 0.1
Gr-22	11.4 ± 0.4** (4.48)	16.1 ± 0.2** (1.54)	6.0 ± 0.3** (1.29)	15.9 ± 0.2** (0.98)	11.9 ± 0.2** (0.98)	6.2 ± 0.1** (0.86)	6.2 ± 0.7** (4.14)	0.2 ± 0.2** (0.33)	19.0 ± 0.1
GrIb-8	7.7 ± 0.4* (6.46)	7.9 ± 0.2* (1.61)	3.7 ± 0.3** (1.70)	7.3 ± 0.2* (0.96)	7.7 ± 0.3* (1.35)	4.7 ± 0.2** (1.40)	3.5 ± 0.2** (4.99)	0** (0)	8.9 ± 0.2
GrIb-9	6.2 ± 0.2** (1.82)	8.5 ± 0.4** (1.57)	4.9 ± 0.3** (2.05)	7.4 ± 0.1** (0.89)	8.6 ± 0.3* (1.37)	4.6 ± 0.2** (1.24)	3.2 ± 0.3** (4.15)	0** (0)	9.8 ± 0.3

[a]TR = (mean colony diameter for fungicide/mean colony diameter for control) mutant/(mean colony diameter for fungicide/mean colony diameter for control) wild-type isolate.

* and ** in each isolate indicate significant differences from control at $P = 0.05$ and $P = 0.01$, respectively.

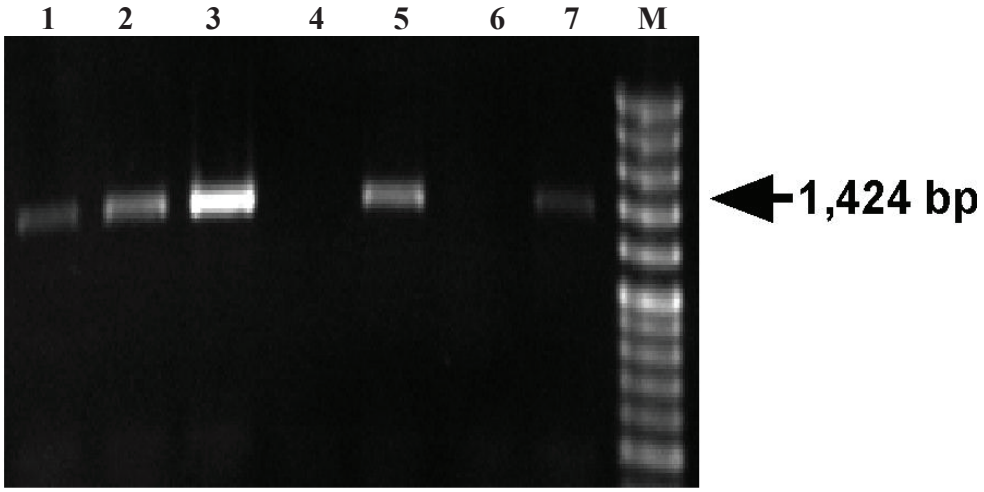

Figure 2. PCR amplification of the *β-tubulin* locus in *I. fumosorosea.* M, DNA ladder marker; lane 1, *I. fumosorosea* PF-3110 (wild-type); lane 2, *I. fumosorosea* GrIb-8; lane 3, *I. fumosorosea* GrIb-9; lane 4, *I. fumosorosea* Ib-34; lane 5, *I. fumosorosea* Gr-22; lane 6, *I. fumosorosea* Gr-5; lane 7, *I. fumosorosea* Ib-421. Arrows indicate predict PCR product sizes.

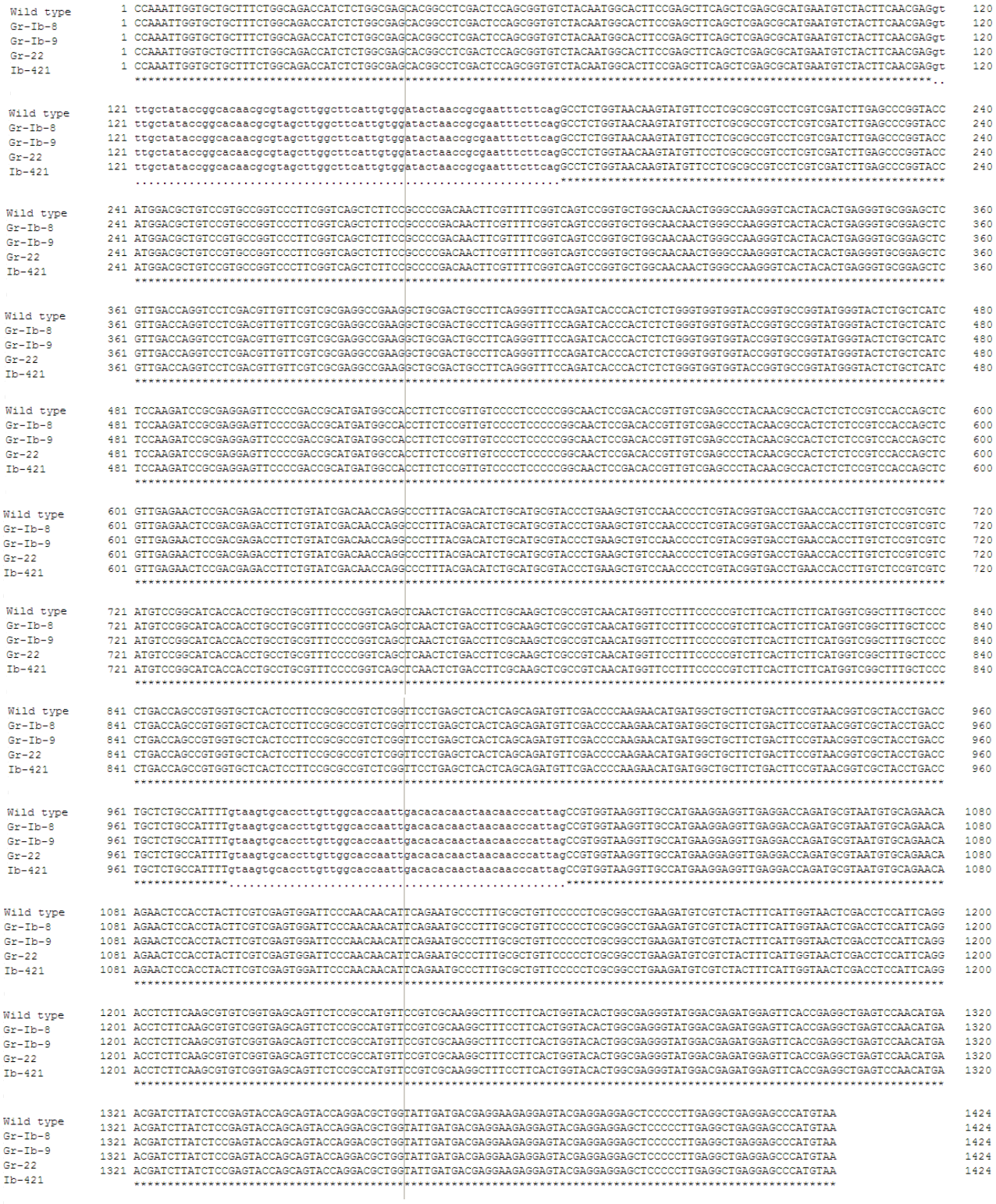

Figure 3. Sequense of *β-tubulin* locus of the wild-type and mutant isolates of *I. fumosorosea*.

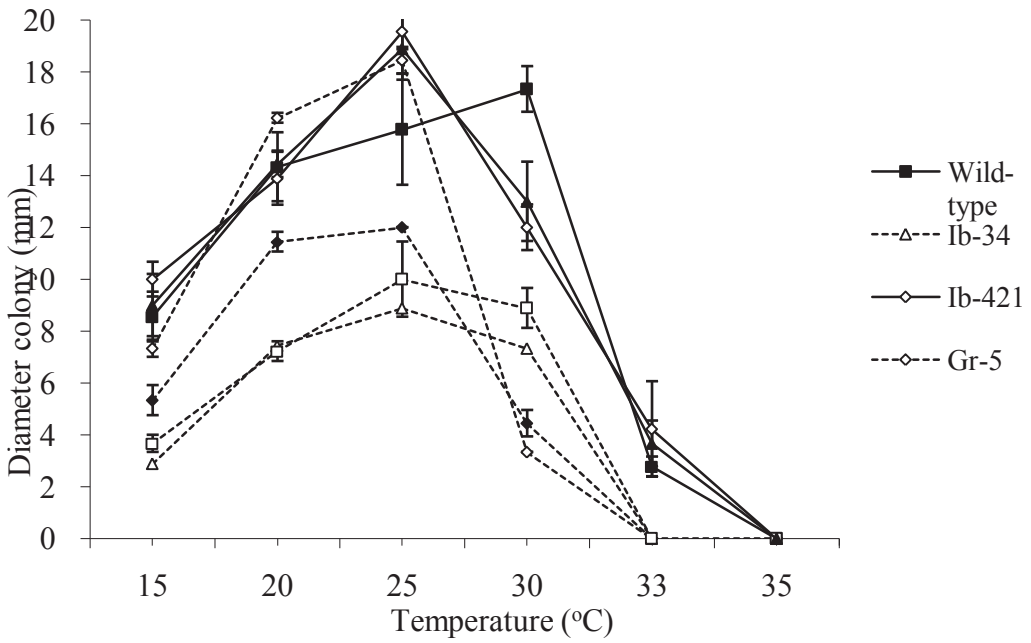

Figure 4. Effect of temperature on mycelial growth of the wild-type and mutant isolates of *I. fumosorosea* on SDA. Bars indicate standard error.

Table 7. Percent mycosis (mean ± SD) of the tobacco whitefly larvae inoculated with the wild-type and mutant isolates of *I. fumosorosea.*

Isolate	Concentration of inoculum (conidia ml^{-1})		
	1×10^6	1×10^7	1×10^8
PF-3110 (wild-type)	97.1 ± 1.9 a	99.9 ± 0.2 a	100 a
Ib-34	63.4 ± 18.0 b	98.0 ± 1.0 a	100 a
GrIb-8	51.1 ± 22.5 b	99.2 ± 0.3 a	99.8 ± 0.3 a
GrIb-9	76.7 ± 4.7 ab	98.4 ± 1.4 a	100 a
Control (water)	0 c	0 b	0 b

The data was convert into Arcsin, Tukey HSD test ($P = 0.05$).
10^6: $F = 40$, $P = <0.0001$
10^7: $F = 616$, $P = <0.0001$
10^8: $F = 4304$, $P = <0.0001$
The number in the same coloumn followed by the same letter is not significantly different.

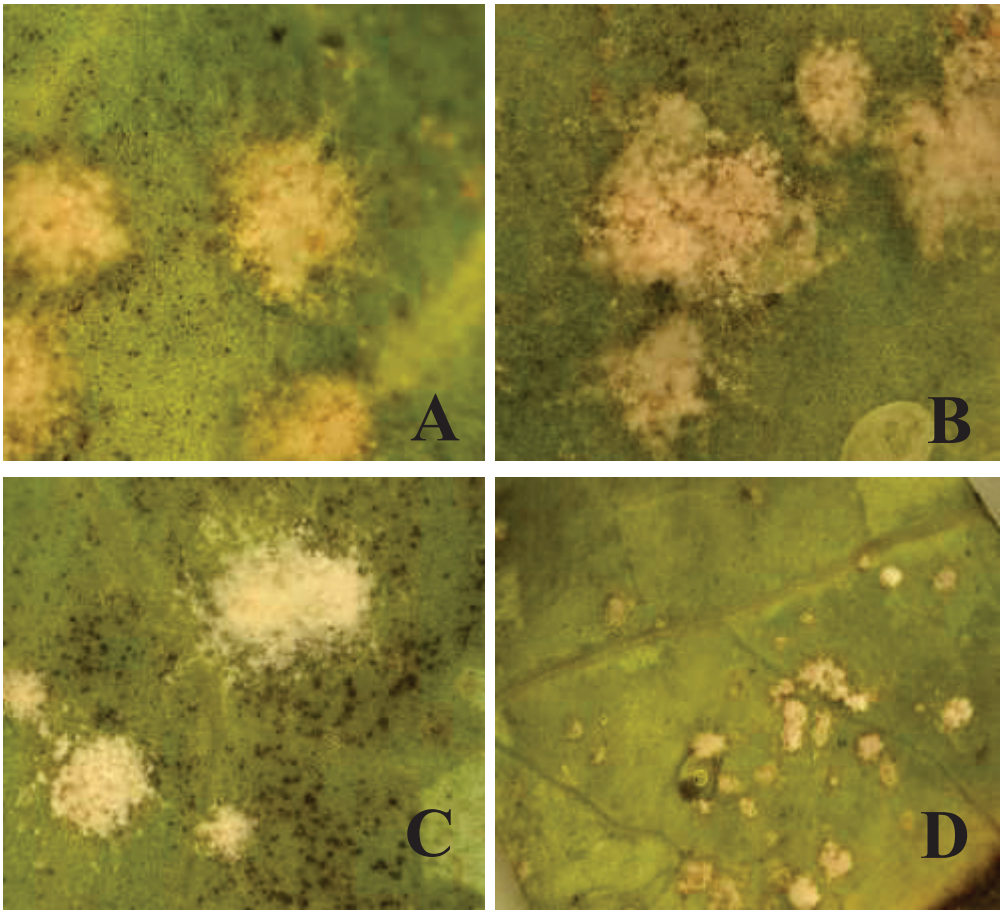

Figure 5. The tobacco whitefly, *B. tabacci* infected by *I. fumosorosea*. A, PF-3110 (Wild-type isolate); B, Ib-34; C, GrIb-8; D, GrIb-9.

CHAPTER 3

ENHANCED FUNGICIDE-RESISTANT IN *BEAUVERIA BASSIANA*

3.1. Introduction

Beauveria bassiana is also important as a biological control agent for many kinds of pest insects belonging to Coleoptera, Hemiptera, Homoptera, Heteroptera, Lepidoptera, and Thysanoptera (Zimmermann, 2007a). One of the examples is use of this fungus to control Thrips (Arthurs et al., 2013; Cloyd, 2009; Saito, 1991). As mentioned in Chapter 1 and 2, *B. bassiana* should also be increasing tolerance to fungicide to a range of commonly used fungicides from other chemical groups for using in IPM programs.

3.2. Materials and Methods

3.2.1. Preparation of fungal material

A stock culture of *Beauveria bassiana* isolate B1026, which is a single-spore strain collected from an unidentified beetle larva in Shizuoka, Japan, in 1991, was used as the wild-type isolate and had been positively identified as *B. bassiana* from the 18S rDNA gene partial sequence (DDBJ/EMBL/GenBank accession number LC008545). For experiments, it was subcultured on to Sabouraud's dextrose agar (SDA, Difco BD Bioscience, USA) in 90 mm diameter Petri dishes, and incubated at 23 ± 1°C for 3 weeks

in darkness. The SDA medium was composed of 40 g dextrose, 10 g peptone, and 15 g agar in 1000 ml distilled water (Goettel and Inglis, 1997). Stock conidial suspensions were prepared by scraping the mycelium from plate cultures into sterile 0.1% Tween 80, agitating with a vortex mixer and then filtering through sterile cloth (0.2 mm mesh size). The concentration of conidia in the stock suspension was determined using a Thoma haemocytometer and adjusted to the concentration required for each experiment by the addition of sterile 0.1% Tween 80.

3.2.2. Fungicides

Seven commercial fungicides were used: benomyl (50% WP, Sumitomo Chemical, Japan), thiophanate-methyl (70% WP, Nippon Soda, Japan), diethofencarb (25% WP, Sumitomo Chemical, Japan), chlorothalonil (40% SC, Kumiai Chemica, Japan), polycarbamate (75% WP, Dow Chemical, Japan), iprodione (50% WP, Nippon Soda), myclobutanil (25% EC, Dow Chemical), and triflumizole (15% EC, Ishihara Sangyo Kaisha, Japan). They are all commonly used fungicides and each came from a different chemical group, expect for benomyl and thiophanate-methyl, which were both benzimidazoles. Each was added to autoclaved SDA once it had cooled to below 50^{o}C and mixed in before pouring into sterile 90 mm diameter Petri dishes (30 ml per dish). The concentration of each fungicides was represented as the active ingredient throughout this study.

3.2.3. Determining effective irradiation doses

Fifty µl of conidial suspension (2×10^3 conidia ml^{-1}) from the wild-type isolate was placed in a Petri dish (60 mm diameter), containing 10 ml of SDA, and spread evenly across the agar surface. Dishes were covered with sterile polyimide film (Kapton 30EN, Du Pont-Toray, Japan) and irradiated at different doses (0, 50, 100, 150, 200, 250, 300, 350 and 400 Gy) with carbon ion beams ($^{12}C^{5+}$, 121.8 keV μm^{-1}, approximately 0.1 to 1 Gy s^{-1}) accelerated by an azimuthally varying field cyclotron at the Takasaki Ion Accelerators for Advanced Radiation Application site (Gunma, Japan). Five replicate dishes were used for each dose. The irradiated dishes, and the conidia they contained, were incubated at 23 ± 1°C in darkness for 3 days. Survival rates were determined by comparing the numbers of colonies per dish (each assumed to originate from one conidium) that grew at each dose and used to select the most appropriate doses to produce mutants.

3.2.4. Selection of benomyl-resistant mutants

Conidia from the wild-type isolate were extracted from 3 ml samples of conidial suspension (1.0×10^8 conidia ml^{-1}) onto sterile cellulose membrane filters (47 mm diameter, 0.45 µm pore size; Millipore, Merck Millipore, Germany) using filtration equipment and washed twice with 10 ml of sterile 0.1% Tween 80. The filters were individually placed in 60 mm Petri dishes and covered with sterile polyimide film. The Petri dishes were then irradiated at the selected doses (see above). Three replicate filters were used for each dose. Each filter was then transferred to a vial containing 3 ml

Sabouraud's dextrose broth (Difco BD Bioscience, USA) and agitated with a sterile glass rod to detach the conidia. The conidial suspensions were incubated at 20 ± 1°C in darkness overnight to remove mutations that were unstable through cell division. Then 200 µl of each suspension was spread onto 30 ml SDA containing benomyl (1000 mg l^{-1}) in a 90 mm Petri dish and incubated at 23 ± 1°C in darkness. Six replicate dishes were made in this way from the contents of each vial. After incubation for 2 weeks, well-grown colonies were assumed to be benomyl-resistant and were isolated onto fresh SDA; colonies that differed in shape and colour from the wild-type isolate were excluded.

3.2.5. Benomyl resistance during vegetative growth

Mycelial plugs (4 mm diameter) from each isolate were excised from the margins of colonies growing on SDA in 90 mm diameter Petri dishes (4–5 days old cultures incubated at 25 ± 1°C in darkness), and each plug was placed upside down at the center of a 90 mm diameter Petri dish containing 30 ml SDA to which benomyl had been added (0.1, 0.3, 1, 3, 10, 30 or 100 mg l^{-1} for the wild-type isolate; 10, 30, 100, 300, 1000 or 3000 mg l^{-1} for the mutants). Nine replicate pairs of treatment and control dishes were prepared for each dose. After incubation at 25 ± 1°C in darkness for 10 days, the colony diameter was estimated from two perpendicular measurements across each colony, excluding 4 mm to account for the diameter of the inoculation plug, and then the mean colony diameter was determined for each replicate. Percent inhabitation values for each replicate were calculated using the formula given below.

Percent inhabitation = (a-b)/a × 100

In this formula, a and b were the colony diameters (mm) of the control and the fungicide dishes from each pair, respectively. The nine data points on percent inhabitation (one from each replicate treatment and control pair of dishes) for each dose were used to estimate the EC_{50} value (50% effective concentration) of each isolate by probit regression analysis against the logarithmic values of the fungicides doses (SPSS, 2009). The tolerance ratio (TR) for each mutant was determined by dividing the EC_{50} value for each mutant isolate by that for the wild-type isolate.

3.2.6. Benomyl resistance during conidial germination

Three 20 µl aliquots of conidial suspension (1.0×10^6 conidia ml^{-1}) from mutant and the wild-type isolates were each placed individually onto 30 ml of SDA containing benomyl (1, 2.5, 5, 10, 25, 50 or 100 mg l^{-1}) in 90 mm diameter Petri dishes. In addition aliquots were placed onto control plates that had been produced in the same way but without fungicide. Each aliquot was covered with a sterile glass coverslip (18 mm × 18 mm). The dishes were incubated at 25 ± 1°C in darkness for 16 h. After the addition of lactophenol cotton blue, the percentage germination of approximately 100 conidia per aliquot was determined under a microscope (Axio Imager 2, Zeiss, Germany). Three replicate dishes were prepared for each dose. The mean percentage germination for three aliquots in each dish was corrected for mean percentage germination in the control (no fungicide) using Abbott's formula (Abbott, 1925), and then the EC_{50} of each isolate was estimated as above, using all data from seven doses.

3.2.7. Tolerance to other fungicides

Tolerance of mutant and the wild-type isolates to seven selected fungicides was estimated from colony diameters on SDA to which the fungicides had been added at recommended field application rates. The methods were as described previously for vegetative growth on agar containing benomyl; nine replicate pairs of treatment and control dishes were prepared for each dose. For each replicate colony vegetative growth ratio (GR) of each isolate was obtained using the following formula.

Colony diameter on fungicide/mean colony diameter on control.

Tolerance to each fungicide was statistically compared using the GRs obtained for each isolate with ANOVA followed by Tukey's HSD test (SPSS, 2009).

3.2.8. *β-tubulin* sequences

Mutant and the wild-type isolates were each cultured on 25 ml SDA at 20°C in darkness for 3 days, and then a small amount of mycelium and conidia from each dish were scraped into a sterile tube for extraction of genomic DNA using a FastDNA Spin Kit (MP Biomedicals, UK). A partial *β-tubulin* sequence was amplified in a GeneAmp PCR System 9700 (Life Technologies, USA) using two specific primers: Beauveria_beta-tub-F and Beauveria_beta-tub-R (Table 8). PCR conditions were an initial 98°C for 20 s, 30 cycles of denaturation at 98°C for 10 s, primer annealing at 63°C for 15 s and extension at 72°C for 2 min followed by a final elongation at 72°C for 5 min. PCR was done in 50 µl reaction volumes consisting of 10 µl genomic DNA (5 ng ml^{-1}), 5 µl 10 × ExTaq buffer, 4 µl dNTP mixture, 0.25 µl ExTaq DNA polymerase (TaKaRa Bio,

Japan), 0.2 µl of each primer (50 µmol l^{-1}) (Sigma Genosys, Japan) and 30.35 µl sterile distilled water. Using these primers produced an amplicon of 1428 bp. The PCR products were run in 0.7% agarose gel made with 1 × Tris-acetate EDTA buffer at 100 V, stained with ethidium bromide (0.5 µg ml^{-1}), and visualized under UV light. The targeted bands were cut from the gels and the PCR product collected and purified using a MinElute PCR Purification Kit (Qiagen, Japan). Purified PCR products were sequenced in an ABI Prism 377 DNA Sequencer (Life Technologies). PCR for sequencing was done in 20 µl reaction volumes containing 2 µl PCR product (20 ng μl^{-1}), 8 µl sterile distilled water, 4 µl 5 × sequencing buffer, 4 µl of primers for *β-tubulin* locus (0.8 µmol l^{-1}) (Table 8) and 2 µl BigDye Terminator v 3.1 (Life Technologies). PCR conditions were 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The products were purified through Sephadex G-50 Superfine gel filtration medium (GE Healthcare, UK), dried at 75°C for 45 min and dissolved in sequence-loading buffer (83% formamide, 4.2 mM EDTA, 8.3 mg ml^{-1} blue dextran). The *β-tubulin* sequences for the mutant isolates were compared with the wild-type isolate sequence in SeqMan Pro (DNASTAR, WI, USA) and GENETYX-MAC (GENETYX, Japan) software packages.

3.2.9. Response of mutants to temperature

We measured colony diameters (as described in 3.2.5) of mutant isolates and the wild-type isolate after 7 days of growth in darkness on SDA in 90 mm diameter Petri dishes (30 ml per dish) at 15, 20, 25, 30, 33 or 35^{o}C. There were three replicate dishes for each isolate at each temperature. At each temperature, the mean colony diameter for each

mutant was compared statistically to the mean colony diameter of the wild-type isolate using the Mann-Whitney *U* test (SPSS, 2009).

3.2.10. Pathogenicity of the mutant isolates

Pathogenicity of mutant isolates and the wild-type isolate were compared against onion thrips, *Thrips tabaci* Lind. (Thysanoptera: Thripidae), as thrips, in general, are a key target pest for development of *B. bassiana* isolate B1026 as a microbial control agent (Saito, 1991). Thrips were supplied by the Kumiai Chemical Company (Japan) and reared on garlic bulbs in containers 17 cm × 24 cm × 9 cm at 25 ± 1^{o}C and 16L: 8D. Groups of ten adults (3 to 5 days old) were transferred to individual petioles of broad bean, *Vicia faba* L. (Fabales: Fabaceae), which were convenient for small scale maintenance of thrips (Murai and Loomans, 2001). For each isolate, three infested petioles were sprayed with a conidial suspension (1.0 × 10^{8} conidia ml^{-1}) using a glass sprayer. The sprayed petioles, and the thrips they harboured, were inserted into glass tubes (18 mm × 50 mm) and incubated at 25 ± 1^{o}C and 16L: 8D. Mortality of adult thrips was recorded daily for 7 days and all cadavers transferred to 60 mm diameter Petri dishes with wet filter paper. The dishes of cadavers were incubated at 25 ± 1^{o}C in darkness to promote fungal development and those developing mycosis recorded. The percentage of mortality caused by mycoses was arcsine square-root transformed and the data for mutant isolates was compared statistically to the data for the wild-type isolate using the Mann-Whitney *U* test (SPSS, 2009).

3.3. Results

3.3.1. Production of benomyl-resistance mutants and tolerance levels

To determine the most appropriate irradiation dose (in Gy, the unit of absorbed energy of ionizing radiation) for production of mutants, we examined the relationship between irradiation dose and conidial survival rate. In previous studies, the highest mutation frequency obtained by ion-beam irradiation was associated with survival rates of 1 to 10% in *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Matuo et al., 2006) and *Aspergillus oryzae* (Ahlburg) Cohn (Toyoshima et al., 2012). Based on this premise, we selected and used doses of 100, 150, 200, 250, 300 and 350 Gy; these doses had resulted in survival rates of between 0.2 to 36.8% in our wild isolate *B. bassiana* (Table 9). We included 100 and 350 Gy even though these doses indicated survival rates outside of the desired 1 to 10% range, because we considered it was safer to have a wide rane and ensure some mutants were produced in the experiments, than use a narrow range and, potentially, produce no mutants (Table 9). Of these doses, 150 Gy provided two large colonies on media containing benomyl (1000 mg l^{-1}) (Figure 6), which were isolated as benomyl-resistant mutants and designated as BB22 and BB24. Conidia without irradiation produced no large colonies on the benomyl-amended media.

In the experiments to determine the EC_{50} for vegetative growth on media amended with benomyl, the wild-type and mutant isolates achieved colony diameters of 35-40 mm on the control plates without fungicide (in 90 mm Petri dishes) after 10-days.

The EC_{50} value of benomyl for vegetative growth was 0.99 mg l^{-1} for the wild-type isolate, 564 mg l^{-1} for BB22 and 828 mg l^{-1} for BB24; TR for the mutants was 570 and 836, respectively (Table 10).

In contrast, there was no significant difference in conidial germination between the wild-type isolate (EC_{50}: 10.2 mg l^{-1}) and the mutants isolates (EC_{50} and TR: 11.6 mg l^{-1} and 1.14 for BB22; and 9.7 mg l^{-1} and 0.95 for BB24) (Table 10).

3.3.2. Tolerance to other fungicides

Tolerance of the wild-type and mutants BB22 and BB24 to seven fungicides at recommended field application rates were compared using GRs. Overall, all isolates tested produced smaller colonies on media with fungicides incorporated than on control media, indicating that the wild-type and the mutant isolates were both negatively affected by these fungicides (Table 11). However, the extent of the effect was different between mutant isolates and the wild-type isolate. On media with benomyl, mutants BB22 and BB24 had significantly larger GRs (0.73 and 0.70, respectively) than the wild-type isolate (GR: 0.12) ($P < 0.05$, Tukey's HSD test) and were, therefore, more tolerant than the wild-type isolate. Both mutant isolates were also significantly more tolerant to thiophanate-methyl (GR: 0.98 and 0.87, respectively) than the wild-type isolate (GR: 0.22) ($P < 0.05$, Tukey's HSD test). Interestingly, the mutant isolates were also significantly more tolerant to chlorothalonil, triflumizole (only BB22) and iprodione (only BB24) than the wild-type isolate ($P < 0.05$). In contrast, on diethofencarb, both BB22 and BB24 had significantly smaller GRs (both 0.34 and 0.35, respectively) than the

wild-type isolate (GR: 0.65) ($P < 0.05$, Tukey's HSD test) and were therefore more sensitive than the wild-type isolate to this chemical.

3.3.3. *β-tubulin* sequences

The *β-tubulin* locus of wild-type and mutant isolates were sequenced to identify mutation sites. A partial genomic DNA sequences of 1428 bp (positions 303 to 1730) (Figure 7) were characterized for all isolates. Isolates BB22 and BB24 both had a single mutation at position 924 (A:T→C:G), which caused an amino acid replacement at position 198 (E→A) (Figure 8). The *β-tubulin* sequence of the wild-type, BB22 and BB24 have been assigned in the DDBJ/EMBL/GenBank with accession numbers AB830334, AB829898 and AB829899, respectively.

3.3.4. Response to temperature

BB22 grew significantly more slowly than the wild-type isolate at 15, 20 and 33°C ($P < 0.05$), and BB24 grew significantly more slowly than the wild-type isolate at all temperatures except 35°C ($P < 0.05$) (Figure 9). Nevertheless, both mutant isolates grew fastest at 25°C, and at that temperature BB22 had a similar growth rate to the wild-type ($P > 0.05$) but BB24 grew significantly more slowly than the wild-type isolate ($P < 0.05$). None of the isolates grew at 35°C.

3.3.5. Pathogenicity of mutant isolates

Mortality in adult thrips began 4 days after inoculation for both the wild-type and mutant isolates treatments. BB22 caused 100% mortality, which was not significantly different from the 89.4% mortality caused by the wild-type isolate ($P > 0.05$). However, BB24 caused only 20.8% mortality, which was significantly less than the wild-type isolate ($P < 0.05$) (Table 12).

3.4. Discussion

Irradiation with ion beams produced benomyl-resistant mutants, as evidenced by their mycelial growth. Such resistance has also been found previously for $NaNO_2$-induced mutants that had a minimal inhibitory concentration (MIC) of > 1000 mg l^{-1} for the benzimidazole fungicide, carbendazim (Zou et al., 2006). These levels of resistance may be sufficient to attenuate for the serious effects of the commercial application rates (250–500 mg l^{-1}) of these fungicides in the field.

The enhanced tolerance may result from cross-resistance between benzimidazole fungicides, such as benomyl and thiophanate-methyl, as seen in plant-pathogenic fungi (Keinath and Zitter, 1998). Interestingly, the mutant isolates were also significantly more tolerant to chlorothalonil, triflumizole (only BB22) and iprodione (only BB24) than the wild-type isolate ($P < 0.05$, Tukey's HSD test), suggesting that the mutant isolates may have multiple mechanisms conferring tolerance to other fungicides that are not benzimidazoles.

This confirmed recent observations for ion beam radiation-induced,

benomyl-tolerant mutants of *I. fumosorosea* in Chapter 2. However, these fungicides would probably still be harmful to the mutant isolates of *B. bassiana* if used practically, because the mutants could only produce very small colonies on media impregnated with them at field rates.

On the contrary, on diethofencarb, both BB22 and BB24 had significantly smaller GRs (0.34) than the wild-type isolate (GR: 0.66) ($P < 0.05$), which meant that these mutants were more sensitive than the wild-type isolate to this chemical. This increased sensitivity may result from negative cross-tolerance between benzimidazole fungicides and *N*-phenylcarbamate fungicides, such as diethofencarb (Fujimura et al., 1992b; Ziogas and Girgis, 1993). Overall, fungicides indicating positive or negative cross-resistance in the present study were similar to fungicides in our recent observations for ion beam irradiation-induced benomyl-resistant mutants of *I. fumosorosea* (Chapter 2).

Thus, *B. bassiana* mutants obtained in this study had resistance during vegetative growth (hyphal extension) but not during initial conidial germination. However, if the mutants are applied at least one day before application of benomyl in the field, they should still successfully control target insect pests, because most conidia germinate within 20 h at 25–32°C and would be growing vegetatively by the time the fungicide was applied (James et al. 1998); infection occurs on the host surface by hyphal extension and penetration of the cuticle (Boucias and Pendland 1998), which would not be affected by the fungicide.

Previous studies have demonstrated that enhanced tolerance to benzimidazole fungicides was associated with a reduction in thermotolerance. For example, carbendazim (a derivative of benzimidazole)-tolerant *B. bassiana* mutants had a shorter LT_{50} (lethal time to kill fifty percent) at a high temperature 48°C compared to the

wild-type isolate (Zou et al., 2006). Such reductions in thermotolerance in entomopathogenic fungi may reduce their potential for use in microbial control. Fortunately, the developed mutants maintained similar thermotolerance characteristics to the wild-type isolate; all had the same optimal temperature (25^{o}C) and upper limit (35^{o}C) for mycelial growth. Further studies are needed to elucidate the mechanisms of thermotolerance and the relationship between benzimidazole-tolerance and thermotolerance in entomopathogenic fungi.

Benzimidazole fungicides bind to *β-tubulin* in the microtubules, thereby inhibiting their proliferation and suppressing dynamic instability (Davidse 1986; Koo et al. 2009). In fact, many mutation sites at the *β-tubulin* locus have been found in benzimidazole-resistant plant-pathogenic fungi (e.g. Albertini et al. 1999; Qiu et al. 2011), but most molecular studies have focused on replacement of the amino acid at position 198 and/or 200 (e.g. Davidson et al. 2006; Fujimura et al. 1992; Hollomon et al. 1998).

Mutation at position 198 in particular resulted in resistance to benzimidazole fungicides in the plant-pathogenic fungi *Monilinia fructicola* (G. Winter) Honey (Ma et al. 2003) and *Venturia inaequalis* (Cooke) G. Winter (Koenraadt et al. 1992). In previous studies on the entomopathogenic fungus *B. bassiana*, mutation at position 198 also led to resistance to benzimidazoles, when glutamate was replaced with lysine (E198K), glycine (E198G), or valine (E198V) (Butters et al. 2003; Zou et al. 2006).

In this study we also found a mutation at position 198 in both mutant isolates, suggesting that mutations at this position are also very important in *B. bassiana*. However, in our study the glutamate was replaced with alanine (E198A). The potential mechanisms for benomyl-resistance may be different between *B. bassiana* and *I.*

fumosorosea, because no mutation at position 198 of the *β-tubulin* locus was detected in benomyl-resistant *I. fumosorosea* mutants produced by ion-beam irradiation.

In conclusion, this study indicates that ion beams are useful tools for enhancing fungicide resistance in *B. bassiana* as has previously been shown for *I. fumosorosea*. Fungicide-resistant mutants produced in this way could be useful agents for biological control within IPM programmes using fungicides, once they have been evaluated for other important characteristics, particularly virulence to target insects.

Table 8. Primers used for *β-tubulin* locus PCR amplification and sequencing of *B. bassiana*.

Primer	Sequence (5′ to 3′)	Use
Beauveria_beta-tub-F	CAGTGCGGTAACCAAATCG	PCR and sequencing
Beauveria_beta-tub-R	CAAAGGCTCCTCGCCCTCAA	PCR and sequencing
Beauveria_beta-tub-F2	CAGGGTTTCCAGATCACCC	Sequencing
Beauveria_beta-tub-R2	CTTCATGGCAACCTTACCAC	Sequencing
Beauveria_beta-tub-F3	GAGGACCAGATGCGTAATGTG	Sequencing
Beauveria_beta-tub-R3	GAACAACGTCGAGGACCTG	Sequencing

Table 9. Relationship between ion-beam irradiation dose and conidial survival rate of wild-type isolates of *B. bassiana*.

Dose (Gy)	No. of colonies per Petri dish (mean ± SEM)	Survival rate[a]
0	193.7 ± 9.8	100
50	145.7 ± 25.0	75.2
100	71.3 ± 15.5	36.8
150	22.0 ± 3.5	11.4
200	7.6 ± 0.3	3.9
250	4.7 ± 0.3	2.4
300	0.7 ± 0.3	0.4
350	0.3 ± 0.3	0.2
400	0	0

[a] Survival rate = (mean number of irradiated colonies/mean number of control colonies) × 100.

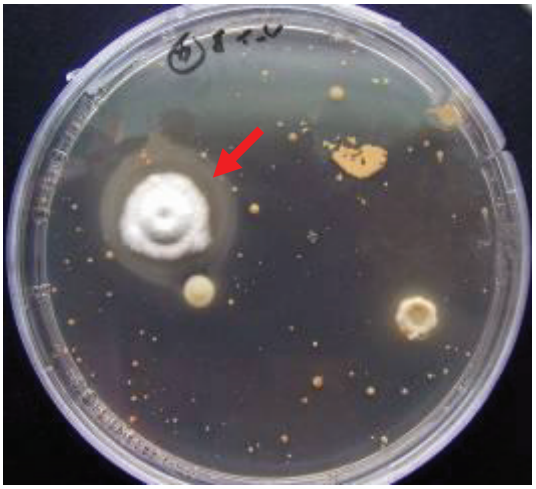

Figure 6. A benomyl-tolerance colony (arrow) derived from *B. bassiana* wild-type isolate conidia irradiated by ion beams (150 Gy) on SDA containing benomyl (1000 mg l^{-1}). Mutants should be indicated by arrows.

Table 10. Benomyl tolerance as measured by vegetative growth and conidial germination of the wild-type and mutant isolates of *B. bassiana* on SDA impregnated with benomyl.

Isolate	Vegetative growth			Conidial germination		
	EC_{50} (mg l^{-1})	95% CL (mg l^{-1})	TR*	EC_{50} (mg l^{-1})	95% CL (mg l^{-1})	TR*
Wild-type	0.99	0.86–1.14	1	10.2	7.6–13.9	1
BB22	564	481–667	570	11.6	9.0–14.9	1.14
BB24	828	634–1137	836	9.7	6.9–14.1	0.95

*TR = EC_{50} value of mutant/EC_{50} value of wild-type isolate.

Table 11. Colony diameters (mm, mean ± SD), with growth ratios (GR*, mean ± SD) in parentheses, of wild-type and mutant isolates of *B. bassiana* on SDA containing different fungicides, each at the recommended field application rate.

Isolate	Benomyl (500 mg l^{-1})	Thiophanate-methyl (700 mg l^{-1})	Iprodione (500 mg l^{-1})	Chlorothalonil (400 mg l^{-1})	Myclobutanil (625 mg l^{-1})	Triflumizole (150 mg l^{-1})	Diethofencarb (250 mg l^{-1})	Control (No fungicide)
Wild-type	4.8 ± 0.6 (0.11 ± 0.01) a	9.4 ± 0.4 (0.22 ± 0.01) a	13.8 ± 1.5 (0.32 ± 0.03) a	37.8 ± 0.6 (0.88 ± 0.03) a	0 (0)	10.8 ± 0.9 (0.25 ± 0.02) a	28.0 ± 1.3 (0.65 ± 0.03) a	43.1 ± 0.1 (1)
BB22	30.2 ± 0.4 (0.73 ± 0.02) b	40.9 ± 0.5 (0.99± 0.02) b	13.7 ± 1.2 (0.33 ± 0.03) a	38.9 ± 0.5 (0.94 ± 0.01) a	0 (0)	15.0 ± 0.6 (0.36 ± 0.02) b	14.0 ± 0.6 (0.34 ± 0.02) b	41.6 ± 0.6 (1)
BB24	27.1 ± 0.3 (0.72 ± 0.01) b	32.6 ± 0.5 (0.87 ± 0.02) c	20.7 ± 0.6 (0.55 ± 0.01) b	33.8 ± 0.7 (0.90 ± 0.02) a	0 (0)	9.7 ± 0.6 (0.26 ± 0.01) a	12.9 ± 0.7 (0.35 ± 0.02) b	37.6 ± 0.2 (1)
	$F_{2,24}$ = 588.3	$F_{2,24}$ = 81.0	$F_{2,24}$ = 26.7	$F_{2,24}$ = 1.6		$F_{2,24}$ = 11.0	$F_{2,24}$ = 60.6	

*GR = (Mean colony diameter for fungicide/mean colony diameter for control) for each replicate.
Mean GRs within a column followed by different lowercase letters are significantly different ($P < 0.05$) according to Tukey's HSD tests.
F values are indicated for each column.

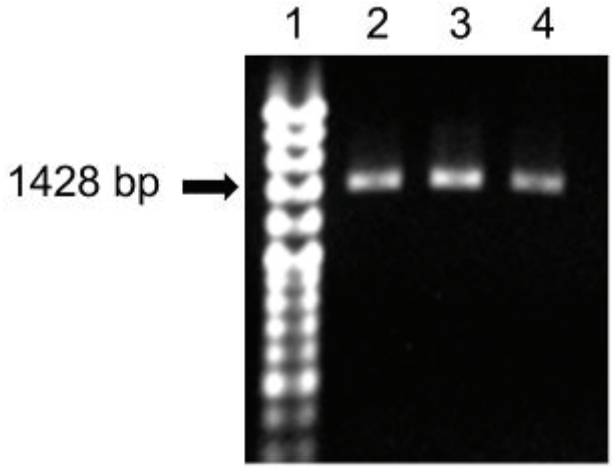

Figure 7. PCR amplification of the *β-tubulin* locus in wild-type and mutant isolates of *B. bassiana*. Lane 1, DNA ladder marker; lane 2, wild-type isolate; lane 3, BB22; lane 4, BB24. Arrow indicates the predicted PCR product size.

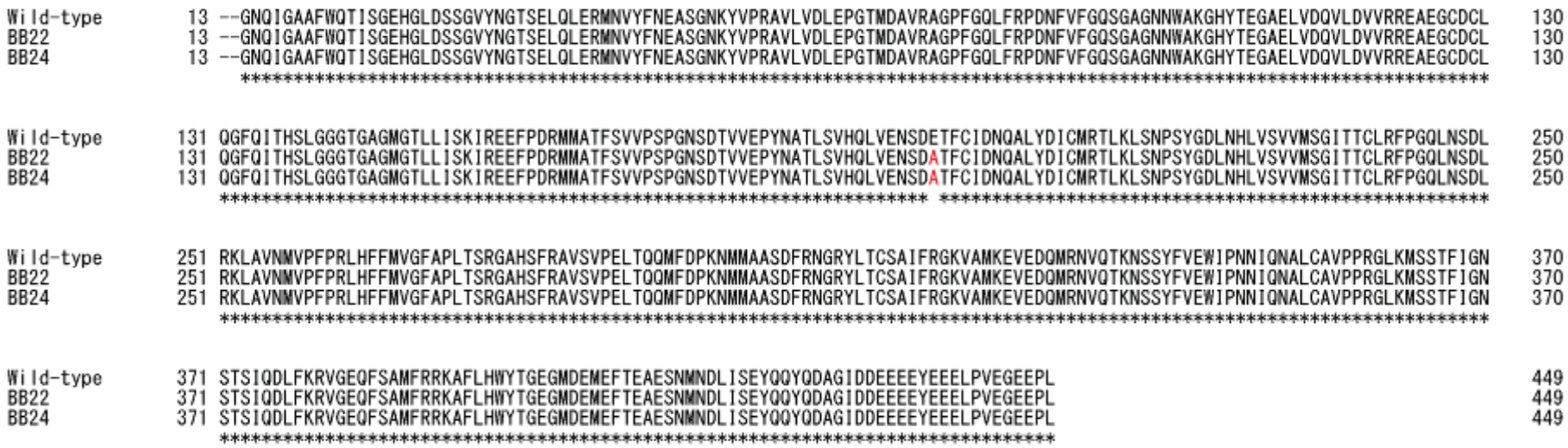

Figure 8. Multiple amino acid sequence alignment of *β-tubulin* locus in the wild-type and mutant isolates of *B. bassiana* by CLUSTAL W (Thompson et al., 1994). Numbers on the left and right indicate sequence positions. Asterisks indicate amino acids conserved in all isolates.

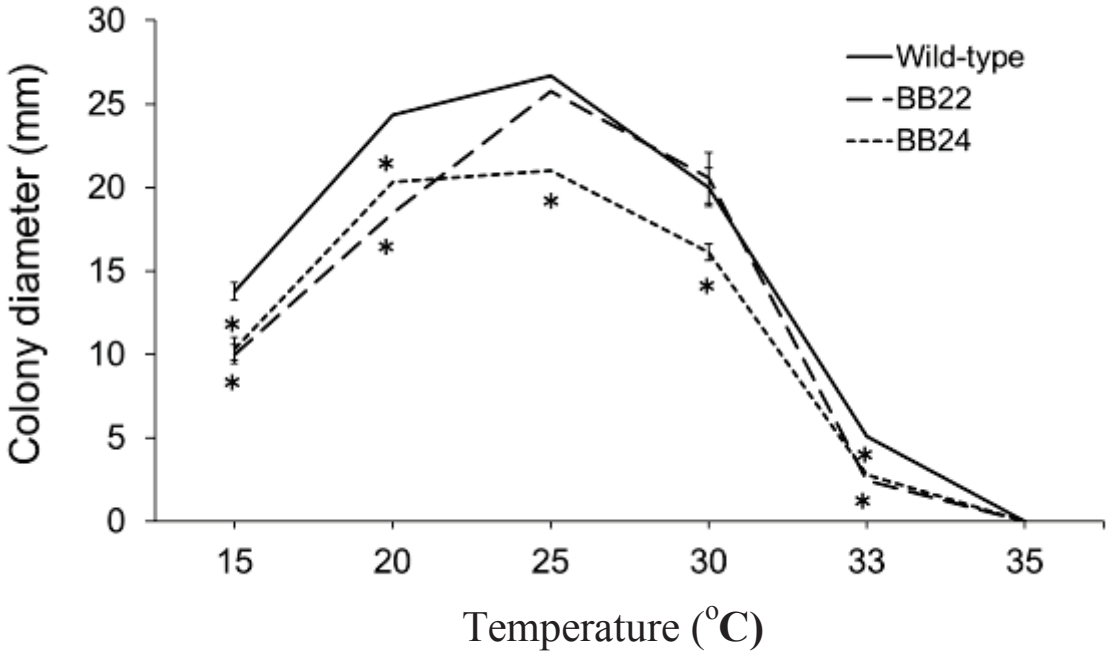

Figure 9. Effect of temperature on mycelial growth of the wild-type and mutant isolates of *B. bassiana* on SDA. Bars indicate standard error. For each temperature, asterisks indicate a significant difference from the wild-type at $P = 0.05$ using the Mann-Whitney *U* test.

Table 12. Mortality due to mycosis of *T. tabaci* adults treated with 1.0×10^8 conidia ml^{-1} of the wild-type and mutant isolates of *B. bassiana*.

Isolate	% Mortality (mean ± SEM)
Wild-type	89.4 ± 0.8
BB22	100
BB24	20.8 ± 5.9 *
Control (water)	0 *

Asterisks indicate significant difference from the wild-type at $P = 0.05$ by Mann-Whitney *U* test.

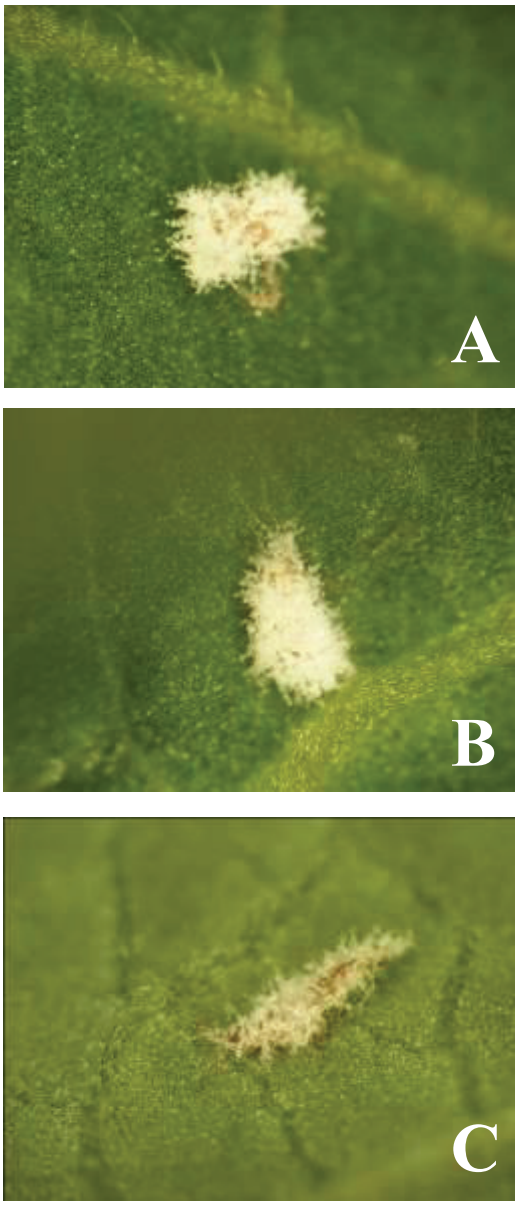

Figure 10. The onion thrips, *T. tabaci* infected by wild-type and mutant isolates of *B. bassiana*. A, B1026 (Wild-type); B, BB22; C, BB24.

CHAPTER 4

ENHANCED THERMOTOLERANCE IN *METARHIZIUM ANISOPLIAE s.l*

4.1. Introduction

Metarhizium anisopliae has been recorded from a wide range of insect pests (Zimmermann, 2007b). Especially this fungus is important as pathogens in tropical pest insects such as locust, grasshopper (Lomer et al., 2001), grubs (Raid and Cherry, 1992; Manisegaran et al., 2011), termites (Hoe et al., 2009), as well as mosquito (Mnyone et al., 2009; Bukhari et al., 2011). The same as other entomopathogenic fungi, *M. anisopliae* is also highly affected by high temperatures (Zimmermann, 2007b). For example, Dimbi et al. (2004) reported that more than 80% of conidia germinated at 20, 25 and 30^{o}C, whereas 26–67% of conidia germinated at 35^{o}C. *M. anisopliae* is a mesophilic fungus which has growth temperature range between 15 and 35^{o}C, and optimum temperature for germination and growth range between 25 and 30^{o}C (Zimmermann, 2007b). Thus, the environment temperature such as more than 35^{o}C generally causes negative effect on this fungus. Thermotolerance of entomopathogenic fungi including *M. anisopliae* become more important since the body temperatures of some insects rise above its ambient temperature as a response to infection of the pathogen (Inglis et al., 1997; Elliot et al., 2002; Springate and Thomas, 2005; Blanford et al., 2009) or when they bask on soil surface under the sunlight (Heinrich and Pantle, 1975; Blanford and Thomas, 2000; O`Neill and Rolston, 2007; Ortiz-Urquiza and Keyhani, 2013). To

use the fungus successfully in biological control of pest insects, we need to develop *M. anisopliae* mutants tolerant to high temperatures.

Some methods have been performed for development of thermotolorant mutants in *M. anisopliae*: automated continuous culture method (de Crecy et al., 2009), artificial selection (Kim et al., 2011), neutral trehalase gene (*Ntl*) over expression (Leng et al., 2011), UV-B radiation (Rangel et al., 2006), nutritional stress, heat shock, osmotic stress (Rangel et al., 2008), and metabolic engineering of dihydroxynaphthalene (DHN) melanin biosynthetic genes (Tseng et al., 2011). However, there is no use of ion beams and gamma rays to develop thermotolerant mutants in this fungus.

4.2. Materials and Methods

4.2.1. Fungal preparation

Two isolates of *M. anisopliae s.l.*, reference numbers AcMa5 and PaMa02, that originated from scarab larvae in Shizuoka, Japan, were used as the wild-type isolates. They were grown on Sabouraud's dextrose agar (SDA; Difco BD Bioscience, USA) in 90-mm diameter Petri dishes and incubated at 23 ± 1°C for 3 weeks in darkness prior to experimentation. Conidial suspensions for experiments were prepared by scraping the mycelium from plate cultures into sterile 0.1% Tween 80 and filtering the resulting suspension of conidia through a sterile cloth (0.2-mm mesh size) to remove mycelia. Conidial concentrations were determined using a Thoma haemocytometer and adjusted as required.

4.2.2. Induction of mutants

For each wild-type isolate, 3 ml samples from the conidial suspension (1×10^8 conidia ml^{-1}) were each passed through replicate 47-mm cellulose membrane filters (pore size 0.45 μm; Millipore, Merck Millipore, Germany). The conidia-laden filters (n = 3 per treatment and irradiation dose) were placed individually in sterile 60-mm plastic Petri dishes and then irradiated with either carbon-ion beams ($^{12}C^{5+}$, 121.8 keV μm^{-1}) accelerated by an azimuthally varying field cyclotron at the Takasaki Ion Accelerators for Advanced Radiation Application site (Gunma, Japan), or gamma rays (^{60}Co, 0.2 keV μm^{-1}) at the Food Irradiation Facility, Japan Atomic Energy Agency (Gunma, Japan). Irradiation doses were 0, 100, 200, 300, 400 or 500 Gy for ion beams and 0, 30, 100, 300, 1000 or 3000 Gy for gamma rays as in Chapter 2. Prior to ion-beam irradiation, the Petri dish lids were replaced with polyimide film (Kapton 30EN, DuPont-Toray, Japan). Each filter was then transferred to a vial containing 3 ml Sabouraud's dextrose broth (Difco BD Bioscience, USA) and agitated with a sterile glass rod to detach the conidia. The conidial suspensions were incubated at 20 ± 1°C in darkness overnight to remove mutations that were unstable through cell division (germination), and then 200 μl of each suspension was spread onto 30 ml SDA in a 90-mm Petri dish and incubated at 38 ± 1°C (a high enough temperature to prevent growth of both wild-type isolates) in darkness for 2 weeks to select for thermotolerant mutants. There were three replicate dishes for each suspension. After this incubation period, any colonies that had grown were assumed to be thermotolerant mutants and were isolated onto fresh SDA for further experiments.

4.2.3. Effect of temperature on vegetative growth of mutant and wild-type isolates

Resulting thermotolerant mutants and the two wild-type isolates were subcultured on SDA in 90-mm Petri dishes and grown for 4–5 days at 25 ± 1°C in darkness. Mycelial plugs (4-mm diameter) from each isolate were then excised from the margins of these colonies, and each plug placed upside down at the centre of a new 90-mm Petri dish containing 30 ml SDA. These plates were incubated at 15, 20, 25, 30, 33, 35, 36, 37, 38 or 39 ± 1°C for 7 days in darkness, after which time the mean colony diameter was determined from two perpendicular measurements of each colony, excluding 4 mm to account for the diameter of the inoculation plug. There were three replicate dishes per temperature for each isolate. The mean colony diameter of each mutant, at each temperature, was compared statistically to that of the wild-type isolate from which it was derived by Mann–Whitney U test (SPSS, 2009).

4.2.4. Characteristic of the mutants

4.2.4.1. Conidial production and size of mutants

Mycelial plugs (4 mm diameter) from each isolate were excised from the margin of colonies growing on SDA in 90 mm diameter Petri dishes (3–4 days old cultures incubated at 25 ± 1^{o}C), and each plug was placed upside down at the center of a 90 mm diameter Petri dishes containing 30 ml SDA. The dishes were incubated at 25 ± 1^{o}C in the darkness for 14 days and then conidia were gently scraped in 30 ml of 0.1% Tween 80 using glass rods. Conidial production for each isolate was counted as number of

conidia containing 20 µl of conidial suspensions using Thoma haemocytometer under a microscope (Axio Imager 2, Zeiss, Germany). The conidial suspension was also used to estimate conidial size of each isolate. Conidial size (length and width) of each isolate was estimated for 100 conidia observed under microscope (Axio Imager 2, Zeiss, Germany).

4.2.4.2. Germination ability of mutants

Conidial suspension (1.0×10^6 conidia ml^{-1}) of the wild-type and mutant isolates was prepared using the method described in 4.2.1. Twenty µl of conidial suspension was put on the 30 ml SDA in petri dishes (9 cm diameter) and cover with a sterile cover glass (18 mm × 18 mm). A petri dish contained 3 patch of conidial suspension and three dishes were used per temperature levels. After incubation at 25, 35 and 38 ± 1^{o}C in the darkness for 18 h, percent germination for about 100 conidia of each inoculum were determined under a microscope (Axio Imager 2, Zeiss, Germany), after additing lactophenol cotton blue solution.

4.2.5. Response to benomyl fungicide

Investigation was performed on the mycelial growth of the wild-type and the mutant isolates on containing benomyl. Benomyl was added into SDA at the 10 ppm (0.02 g l^{-1}), 1 ppm (0.002 g l^{-1}) and control (0 ppm) after cooling down at about 50^{o}C. Five petri dishes were used. The petri dishes were inoculated with a mycelial plugs (4 mm diameter), which were bored from the margin of actively growing colonies on the SDA

after 3–4 days incubation at 25^{o}C and incubated at 25 ± 1^{o}C in the dark condition for 12 days.

4.2.6. Heat shock stress on conidial germination of mutant and wild-type isolates

This experiment was done following the method of Leng et al. (2011) with some modification as described below. For wet-heat shock test, each mutant and wild-type isolate, 20 ml samples of conidial suspension (1×10^7 conidia ml^{-1}), prepared as described previously, were placed into individual 50 ml flasks and incubated in a stirred water bath at 45 ± 1°C. For the dry-heat shock test, conidia were dried in a desiccator containing silica gel until the moisture content was less than 5%. Dried conidia were maintained in an incubator at 65^{o}C. After 0, 0.5, 1 and 3 h, 50 µl samples were taken from each flask (for dry heat-shock test, the samples were suspended in sterilized water) and inoculated at three positions in each of three 90-mm Petri dishes, each containing 30 ml SDA (i.e. nine 50 µl samples taken from each flask at each time). These plates were incubated at 25 ± 1°C for 24 h, after which time the mean germination rate was determined for approximately 100 conidia from each position (total = approximately 300 per plate) under a microscope (Axio Imager 2, Zeiss, Germany). The mean germination rates for each treatment, at each incubation time, were statistically analysed using ANOVA followed by Tukey's HSD test and Student's t-test for multiple comparisons of means and single comparisons of means, respectively (SPSS, 2009).

4.2.7. Sequences

4.2.7.1. *Ntl* locus

A *Ntl* sequence was amplified in a GeneAmp PCR System 9700 (Life Technologies, USA) using two specific primer sets: Metarhizium_Ntl-F1 and Metarhizium_Ntl-R1 (Table 13). PCR conditions were an initial 98^{o}C for 20 s, 30 cycles of denaturation at 98^{o}C for 10 s, primer annealing at 55^{o}C for 15 s and extension at 72^{o}C for 2.5 min followed by a final elongation at 72^{o}C for 7 min. PCR was done in 50 µl reaction volumes consisting of 5 µl genomic DNA (10 ng ml^{-1}), 5 µl 10 × ExTaq buffer, 4 µl dNTP mixture, 0.25 µl ExTaq DNA polymerase (TaKaRa Bio, Japan), 0.2 µl of each primer (50 µM) (Operon Biotechnologies, Japan) and 35.35 µl sterile distilled water. Using these primers produced an amplicon of 2355 bp. The PCR product was purified using a MinElute PCR Purification Kit (Qiagen, Japan). Purified PCR products were sequenced in an ABI Prism 377 DNA Sequencer (Life Technologies). PCR for sequencing was done in 20 µl reaction volumes containing 3 µl PCR product (20 ng µl^{-1}), 7 µl sterile distilled water, 4 µl 5 × buffer, 4 µl of primers for *Ntl* locus (0.8 µM) (Table 13) and 2 µl BigDye Terminator v 3.1 (Life Technologies). PCR conditions were 25 cycles at 96^{o}C for 10 s, 50^{o}C for 5 s and 60^{o}C for 4 min. The products were purified through Sephadex G-50 Superfine gel filtration medium (GE Healthcare, UK), dried at 75^{o}C for 45 min and dissolved in sequence-loading buffer (83% formamide, 4.2 mM EDTA, 8.3 mg ml^{-1} blue dextran).

4.2.7.2. *β-tubulin* locus

A *β-tubulin* sequence was amplified in a GeneAmp PCR System 9700 (Life Technologies, USA) using two specific primers: Metarhizium_beta-tub-F1 and Metarhizium_beta-tub-R1 (Table 14). PCR conditions were an initial 98^{o}C for 20 s, 30 cycles of denaturation at 98^{o}C for 10 s, primer annealing at 63^{o}C for 15 s and extension at 68^{o}C for 2 min followed by a final elongation at 68^{o}C for 5 min. PCR was done in 50 µl reaction volumes consisting of 5 µl genomic DNA (10 ng ml^{-1}), 10 µl 5 × PrimeSTAR GXL buffer, 4 µl dNTP mixture, 1 µl PrimeSTAR GXL DNA polymerase (TaKaRa Bio, Japan), 0.3 µl of each primer (50 µM) (Operon Biotechnologies, Japan) and 29.4 µl sterile distilled water. Using these primers produced an amplicon of 1901 bp. The PCR product was purified using a MinElute PCR Purification Kit (Qiagen, Japan). Purified PCR products were sequenced in an ABI Prism 377 DNA Sequencer (Life Technologies). PCR for sequencing was done in 20 µl reaction volumes containing 2 µl PCR product (20 ng µl^{-1}), 8 µl sterile distilled water, 4 µl 5 × buffer, 4 µl of primers for *β-tubulin* locus (0.8 µM) (Table 14) and 2 µl BigDye Terminator v 3.1 (Life Technologies). PCR conditions were 25 cycles at 96^{o}C for 10 s, 50^{o}C for 5 s and 60^{o}C for 4 min. The products were purified through Sephadex G-50 Superfine gel filtration medium (GE Healthcare, UK), dried at 75^{o}C for 45 min and dissolved in sequence-loading buffer (83% formamide, 4.2 mM EDTA, 8.3 mg ml^{-1} blue dextran).

4.2.7.3. *ifT1* locus

A *ifT1* sequence was amplified in a GeneAmp PCR System 9700 (Life Technologies, USA) using two specific primer sets: Metarhizium_ifT1-F1 and Metarhizium_ifT1-R8 and Metarhizium_ifT1-F9 and Metarhizium_ifT1-R1 (Table 15). PCR conditions were an initial 98^{o}C for 20 s, 30 cycles of denaturation at 98^{o}C for 10 s, primer annealing at 60^{o}C for 15 s and extension at 72^{o}C for 4.5 min followed by a final elongation at 72^{o}C for 7 min. PCR was done in 50 µl reaction volumes consisting of 5 µl genomic DNA (10 ng ml^{-1}), 5 µl 10 × ExTaq buffer, 4 µl dNTP mixture, 0.25 µl ExTaq DNA polymerase (TaKaRa Bio, Japan), 0.2 µl of each primer (50 µM) (Operon Biotechnologies, Japan) and 35.35 µl sterile distilled water. Using these primer sets produced amplicons of 4211 and 3493 bp, respectively. The PCR product was purified using a MinElute PCR Purification Kit (Qiagen, Japan). Purified PCR products were sequenced in an ABI Prism 377 DNA Sequencer (Life Technologies). PCR for sequencing was done in 20 µl reaction volumes containing 3 µl PCR product (20 ng µl^{-1}), 7 µl sterile distilled water, 4 µl 5 × buffer, 4 µl of primers for *ifT1* locus (0.8 µM) (Table 15) and 2 µl BigDye Terminator v 3.1 (Life Technologies). PCR conditions were 25 cycles at 96^{o}C for 10 s, 50^{o}C for 5 s and 60^{o}C for 4 min. The products were purified through Sephadex G-50 Superfine gel filtration medium (GE Healthcare, UK), dried at 75^{o}C for 45 min and dissolved in sequence-loading buffer (83% formamide, 4.2 mM EDTA, 8.3 mg ml^{-1} blue dextran).

4.2.8. Virulence of mutant and wild-type isolates

Virulence of mutant and wild-type isolates was compared against maize weevils, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), that had been reared on rice grains at 25 ± 1°C and a photoperiod 16L: 8D. Groups of 10 adults (3–7 days old) were placed in glass tubes (20-mm diameter, 30 mm high) from which the bottom had been removed and replaced with gauze. Each group was dipped into a conidial suspension (1×10^7 conidia ml^{-1}), prepared as described previously, through the gauze for 10 s and then incubated on rice grains in a Petri dish (60 mm) with a moist filter paper (to maintain a high humidity) at 25, 30 or 35 ± 1°C with a photoperiod 16L: 8D. Five replicate groups of weevils were used for each isolate with an additional five control groups that were treated in the same way but only with 0.1% Tween 80 and no inoculum. Mortality was recorded daily for 10 days. Dead adults were transferred to Petri dishes lined with moist filter paper and incubated at 25 ± 1°C in darkness for 5 days to allow fungal outgrowth to occur if it had been the cause of mortality. The percentage mortality due to fungal infection was arcsine square-root transformed and then the mean mortalities, at each day after inoculation, were statistically analysed using ANOVA followed by Tukey's HSD test and Student's t-test for multiple comparisons and single comparisons, respectively (SPSS, 2009).

4.3. Results

4.3.1. Production of thermotolerant mutants

Thermotolerant mutants were successfully isolated by screening at 38°C which completely inhibited vegetative growth of the wild-type isolates (Table 16; Figure 11). Four mutants were isolated from the wild-type isolate, AcMa5; one mutant (AcMa5-ib) was from ion-beam irradiation and three mutants (AcMa5-gr-1, AcMa5-gr-2 and AcMa5-gr-3) were from gamma-ray irradiation. From another wild-type isolate, PaMa02, one mutant (PaMa02-ib) was isolated as a result of ion-beam irradiation and no mutants were obtained from gamma-ray irradiation.

4.3.2. Response to some temperatures

Vegetative growth of the mutant isolates at different temperatures was compared to that of the wild-type isolates (Table 17). All the mutant isolates derived from wild-type isolate AcMa5 had an upper thermal limit of 38°C which was higher than that (36°C) of the wild-type isolate by 2°C. Of the mutants, at each temperature from 25°C to 36°C, three mutants (AcMa5-ib, AcMa5-gr-2 and AcMa5-gr-3) also grew significantly faster than the wild-type isolate ($P < 0.05$, Mann–Whitney U test). In contrast, mutant isolate PaMa02-ib derived from wild-type isolate PaMa02 had an upper thermal limit of 39°C which was higher than that (36°C) of the wild-type isolate by 3°C, although this mutant grew significantly slower than the wild-type isolate at 25°C which was the optimal temperature for vegetative growth of both mutant and wild-type isolates ($P < 0.05$,

Mann–Whitney U test). From observations of the colonies growing at each temperature it was notable that, at 35°C, neither the wild-type isolates nor the mutant isolate PaMa02-ib produced any conidia.

4.3.3. Characteristic of the mutants

Both of the wild-type isolates showed yellow colony color at 35 and 36°C, however, all of the mutants showed green and pale green colony at 35 and 36°C, respectively. In contrast, all of the mutants had yellow colony at 37 and 38°C. The conidia were still found in the green, but none in the yellow colony (Table 18). All the mutants of AcMa5 produced much more conidia than the wild-type isolate but not for the mutant of PaMa02, that was the same as the wild-type isolate (Table 18). The size of the conidia (length and width) of the entire mutants of both PaMa02 and AcMa5 were similar as the wild-type isolates (Table 18).

The conidia produced by all of mutants and the wild-type isolates germinated at 25°C (71.05–92.14%) and 35°C (51.43–86.34%) but none of the wild-type isolates and the mutants germinated at 38°C, with one exception. The conidia produced by one mutant of AcMa-5 (AcMa5-gr-1) germinated at 38°C even only 20.14% (Figure 12).

4.3.4. Response to benomyl fungicide

All the mutants were able to grow at 1 ppm of benomyl, however only AcMa5-gr-3, PaMa02-ib and AcMa5 (wild-type isolate) were able to grow at 10 ppm but none for the

other mutants. The colony diameter of AcMa5-gr-3 was significantly wider than its wild-type isolate (AcMa5) and PaMa02-ib (Figure 13).

4.3.5. Heat shock treatment

The results of wet-heat shock treatment are shown in Table 19. Conidia of the wild-type isolate (AcMa5) were unable to germinate after 1 h exposure, whereas all the mutants derived from it germinated (5.6–19.3%). Of these mutants, AcMa5-ib had significantly greater germination rates than other mutants after both 0.5 h ($F = 34.9$; df = 4,10; $P < 0.05$, Tukey's HSD test) and 1 h exposure ($F = 50.0$; df = 4,10; $P < 0.05$, Tukey's HSD test). Thus, mutant isolates derived from AcMa5 had enhanced thermotolerance in respect to conidial germination, though no mutant isolates survived the longest exposure period (3 h). By contrast, the mutant PaMa02-ib derived from wild-type isolate PaMa02 was, like the wild-type isolate, unable to germinate after 1 h exposure, and also, its germination rate was not significantly different to the wild-type isolate after 0.5 h exposure ($P > 0.05$, Student's t-test).

No germination was found on both of the wild-type and all mutants isolates when incubated at 60^{o}C (dry heat) for 1 h. At 0.5 h, the wild-type isolate (AcMa5) and AcMa5-gr-1, showed no germination. The other mutants showed germination 29.88–81.25% (Table 20).

4.3.6. Sequence analysis of *Ntl*, *β-tubulin* and *ifT1* locus

The mechanisms for enhanced thermotolerance in the mutants are unknown although initial studies (Figure 14; Figure 15 and Figure 16) suggest that there were no mutations in the neutral trehalase gene (*Ntl*), *β-tubulin* gene or the ABC transporter gene (*ifT1*) previously associated with thermotolerance and fungicide tolerance in entomopathogenic fungi (Leng et al., 2011; Song et al., 2011; Zou et al., 2006). This requires further research.

4.3.7. Pathogenicity of mutants

In this study, each mutant isolate was also evaluated for virulence to weevil adults at a standard temperature (25°C) and at higher temperatures (30 and 35°C). At 25°C, all the mutants derived from wild-type isolate AcMa5 caused high mortalities (86–100%) at Day 10, which were not significantly different from the mortality (90%) caused by the wild-type isolate (F = 1.5; df = 4,20; P > 0.05, Tukey's HSD test; Figure 17A). By contrast, the mutant PaMa02-ib derived from wild-type isolate PaMa02 caused significantly less mortality (4%) at Day 10 than the wild-type isolate (94%; P < 0.05, Student's t-test; Figure 17B). At 30°C, there was no significant difference in mortality at Day 10 amongst wild-type isolate AcMa5 and the four mutants derived from it (F = 2.1; df = 4,20; P > 0.05, Tukey's HSD test), though there was a trend for AcMa5-ib to cause higher mortality compared to the wild-type isolate throughout the experiment (e.g. 84% mortality compared with 58%, respectively; Figure 18A). Mutant isolate PaMa02-ib derived from wild- type isolate PaMa02 caused significantly less mortality (6%) at Day

10 than the wild type (30%; $P < 0.05$, Student's t-test; Figure 18B). Throughout the bioassays at 25°C and 30°C, no mortality was observed in control weevils. At 35°C, all mutants and wild-type isolates caused some fungal-induced mortality, however, the weevils lost their appetite and a number died without obvious signs of fungal infection in both the treatments (18–32%) and control (24%). Since such high temperatures were harmful to the weevils, we did not used data from the 35°C treatment in the analysis. The virulence tests showed that most mutant isolates retained high levels of virulence at the standard temperature (25°C), and one mutant (AcMa5-ib) generated by ion beams was apparently more virulent than the wild type at a higher temperature (30°C).

4.4. Discussion

Ion-beam and gamma-ray irradiations have been reported as useful method to improve traits of organism through mutagenesis (Matuo et al., 2006; Tanaka et al., 2010; Toyoshima et al., 2012). For example, using ion-beam irradiation, Das et al. (2008, 2010) successfully developed mutant of *Cordyceps militaris* which capable of enhanced production of cordycepin, a medicinal adenosine analogue. However, there are limited reports on the use of ion-beam and gamma-ray irradiations for mutant development in entomopathogenic fungi.

Using ion-beam or gamma-ray irradiations, we have developed 5 mutants derived from two wild-type isolates i.e. AcMa5 and PaMa02. One mutant AcMa5-ib was generated from ion-beam irradiation and three mutants i.e. AcMa5-gr-1, AcMa5-gr-2 and AcMa5-gr-3 were generated from of gamma-ray irradiation (Table 16) and one mutant PaMa02-ib was also derived from ion-beam irradiation (Table 16). All the mutants

resulted different traits in responds to temperature, even the mutants derived from the same wild-type isolate and or the same irradiation.

Three mutants (AcMa5-ib, AcMa5-gr-2 and AcMa5-gr-3) derived from wild-type isolate (AcMa5) grew significantly faster than the wild-type isolate and another mutant AcMa5-gr-1 ($P < 0.05$, $F_{4,10} = 219.8$, Tukey HSD test, Table 17). In contrast, a mutant PaMa02-ib generated from wild-type isolate (PaMa02) grew significantly slower than the wild-type isolate ($P = 0.016$, $F = 16.0$, Student *t*-test, Table 17). This means that we cannot predict traits resulted by mutagenesis derived from the same irradiation method. In line with the statement of Shapiro-Ilan et al. (2011), we agree that resulting mutants should be carefully evaluated for unpredictable negative effects.

Thermotolerant mutants of *M. anisopliae* were also developed by de Crecy et al. (1999) using continous culture method. They produced thermotolerance mutant with upper thermal limit at 38^{o}C. The mutant developed in this study showed 1^{o}C higher of upper thermal limit than of the mutant developed by de Crecy et al. (1999).

Both of the wild-type isolates showed yellow colony color at 35 and 36^{o}C, however, All the mutants generated from AcMa5 (AcMa5-ib, AcMa5-gr-2 and AcMa5-gr-3) produced characteristic green conidia at temperatures of 35°C and produced much more conidia than the wild-type isolate, suggesting that these mutants were better adapted to high temperatures in respect of their sporulation capacity than the wild-type isolate. On the contrary, the mutant generated from PaMa02 (PaMa02-ib) produced characteristic yellow conidia at temperature 35°C and produced the same amount of conidia as the wild-type isolate, suggesting that this mutant was in the same tolerance to high temperature as the wild-type isolate. However, all of the mutants showed yellow colony at 37 and 38^{o}C. The conidia were still found in the green, but none in the yellow colony

(Table 18). It is showed that the mutants improved their tolerance to produce conidia in high temperature stress condition.

The size of the conidia (length and width) of the entire mutants of both PaMa02 and AcMa5 were same as the wild-type isolate. It showed that ion-beam and gamma-ray irradiations did not change the conidial size of the mutants but significantly enhanced conidial production of the mutants of AcMa5, however not for PaMa02. However, after mutation, conidial production of thermotolerance mutants will not always enhanced. For example, thermotolerance mutants developed by de Crecy et al. (1999) have lower (EVG017) and same (EVG017g) ability to produce conidia as the wild-type isolate.

Thermotolerance in conidia of the mutants may represent a considerable advantage for their practical use in biological control because conidial germination is the first step in the infection process of host insects (Boucias and Pendland, 1998; Castrillo et al., 2005). Thus, we also checked germination of the conidia produced by all of the mutants in different temperature conditions. de Crecy et al. (2009) reported that *M. anisopliae* variant generated by natural selection–adaptation was able to germinate and grow well at 37°C; in contrast, while the wild-type isolate from which it was derived was able to germinate at 37°C, it failed to subsequently grow. Their findings are similar to the results for vegetative growth in mutant and wild-type isolates. The mutants and the wild-type were able to germinate at 25^{o}C (71.05–92.14%) and 35^{o}C (51.43–86.34%), however none of the wild-type isolates and the mutants germinated at 38^{o}C, except AcMa-5-gr-1. This mutant was germinated at 38^{o}C even only 20.14% (Figure 12).

In a study by Leng et al. (2011), mutant isolates of *M. acridum* (Driver and Milner) Bischoff, Rehner and Humber (Hypocreales: Clavicipitaceae) generated by neutral trehalase gene RNA interference transformations, were significantly more tolerant to

wet–heat stress at 45°C for 1 , 1.5 , 2 , 2.5 and 3 h exposure than the wild-type isolate from which they were derived. The mutant isolates of *M. acridum* remained able to germinate at low levels (< 20%) after 3 h. There is no mutant isolates survived in the longest exposure period (3 h). Using their method, similar results were obtained in this study. When each conidia suspension was incubated at 45^{o}C for 1 h followed by observation for germination rate on SDA at 25^{o}C, wild-type isolate AcMa5 revealed no germination whereas the mutants exhibited a range of 7.5–19.3% for germination, although another wild-type isolate PaMa02 and the mutant PaMa02-ib showed no germination (Table 19). These findings suggest that most mutants also enhanced thermotolerance in conidial production and persistence which are important factors for infection on target insects (Castrillo et al., 2005). Thus both ion-beam and gamma-ray irradiations successfully developed mutants more tolerant to heat stress in not only vegetative growth but also conidial germination of *M. anisopliae*.

Previous studies have reported that decrease on thermotolerance was correlated with the enhanced tolerance to benzimidazole fungicide. As reported by Zou et al. (2006), carbendazim-tolerant *B. bassiana* mutants had a shorter LT_{50} (lethal time to kill fifty percent) at a high temperature 48^{o}C compared to the wild-type isolate (Zou et al., 2006). Thus such correlation may have occurred in the developed thermotolerance mutants. Unfortunately, the developed thermotolerance mutants did not show either positive or negative correlation with their tolerance to benzimidazole fungicide. Each of the mutant gave varied result on the tolerance to benzimidazole fungicide (Figure 13). It need further study on the correlation between thermotolerance and benzimidazole tolerance of entomopathogenic fungi.

Even all the mutant developed in this study showed different traits from their wild-type isolate, they had identical sequence of nucleic acid of several genes. The mutants had no mutations in the neutral trehalase locus (*Ntl*) (Leng et al., 2011), *β-tubulin* locus (Zou et al., 2006; Song et al., 2011) and the ABC transporter locus (*ifT1*) (Song et al., 2011), respectively. Further studies are needed for the mechanisms resulting in enhanced thermotolerance in the developed mutants.

Most mutants retained original virulence of wild-type isolate, though one mutant greatly declined virulence. All the mutants from wild-type isolate AcMa5 exhibited final mortalities which was not significantly different from the wild-type isolate (Figure 17) at 4 d and 10 d. Here, a negative effect of mutagenesis on the resulting mutant was revealed. The mutant PaMa02-ib derived from another wild-type isolate PaMa02 resulted a final mortality which was significantly less than caused from the wild-type isolate. Unpredictable negative effect of mutagenesis was also reported by Shapiro-Ilan et al. (2011).

As mentioned above, some mutants of *M. anisopliae* have enhanced tolerance to heat stress in vegetative growth, sporulation and germination. The results suggest that ion beams and gamma rays are a useful tools for improving thermotolerance in *M. anisopliae* and thereby increasing the potential for application of entomopathogenic fungi as microbial control agents. This is the first report of enhanced thermotolerance induced by ion beams or gamma rays in entomopathogenic fungi. However, we also found that some thermotolerant mutants generated by ion beams, included mutant isolate AcMa5-ib, almost lost their virulence entirely. This finding suggests that thermotolerance does not always promise higher virulence at a high temperature range. The resulting mutant

isolates should be carefully evaluated for unpredictable negative effects before practical use is possible (Shapiro-Ilan et al., 2011).

Table 13. Primers used for *Ntl* locus PCR amplification and sequencing of *M. anisopliae*.

Primer	Sequence (5′ to 3′)	Use
Metarhizium_Ntl_F1	ATGGCGGGAACGACAAAC	PCR and sequencing
Metarhizium_Ntl_F2	GAAGACACCGATGGCAATATGC	Sequencing
Metarhizium_Ntl_F3	GTCTAGATGTCCAGCAGCTC	Sequencing
Metarhizium_Ntl_F4	CTTAGACGGTCGATACTAGCTG	Sequencing
Metarhizium_Ntl_F5	ACGACAAGCTTGTGATCCC	Sequencing
Metarhizium_Ntl_F6	GGTATAGCTTCACAGAAGAGGC	Sequencing
Metarhizium_Ntl_R1	CTCTGTTCAATCGCCTTGATAAAC	PCR and sequencing
Metarhizium_Ntl_R2	CAAGCATCTGCTGAGGAGC	Sequencing
Metarhizium_Ntl_R3	GCGCAATGTCGGTTTCG	Sequencing
Metarhizium_Ntl_R4	GGCATCCGGTTCATGCTT	Sequencing
Metarhizium_Ntl_R5	CTCCTCGGCAACTTGCTT	Sequencing
Metarhizium_Ntl_R6	CTTTCGAGAGTAGGGTCGAC	Sequencing

Table 14. Primers used for *β-tubulin* locus PCR amplification and sequencing of *M. anisopliae.*

Primer	Sequence (5′ to 3′)	Use
Metarhizium_beta-tub-F1	TGAAGCTCTGCCTAGCTATC	PCR and sequencing
Metarhizium_beta-tub-F2	CCAAATTGGTGCTGCTTTCTGG	Sequencing
Metarhizium_beta-tub-F3	GTCCTTGATGTTGTCCGTCG	Sequencing
Metarhizium_beta-tub-F4	GATCTGCGTAAGCTGGCTGTC	Sequencing
Metarhizium_beta-tub-F5	GGCCTCAAGATGTCTTCTAC	Sequencing
Metarhizium_beta-tub-R1	CAAGTCGGACATTCTAGCTG	PCR and sequencing
Metarhizium_beta-tub-R2	TGCACGTTACGCATCTGGTC	Sequencing
Metarhizium_beta-tub-R3	GTTAGACAGCTTGAGAGTGC	Sequencing
Metarhizium_beta-tub-R4	GAAAAGCTGACCGAAAGGAC	Sequencing
Metarhizium_beta-tub-R5	GTTTACTTACGCACTGGCC	Sequencing

Table 15. Primers used for *ifT1* locus PCR amplification and sequencing of *M. anisopliae*.

Primer	Sequence (5′ to 3′)	Use
Metarhizium_ifT1_F1	GGCCTAGGAAGCACGAA	PCR and sequencing
Metarhizium_ifT1_F2	CAGAGTGCCCACAGCTG	Sequencing
Metarhizium_ifT1_F3	CATGCGCTTGGGTACGCT	Sequencing
Metarhizium_ifT1_F4	ACCATCAGCGCTAATACGGAC	Sequencing
Metarhizium_ifT1_F5	GTTGGTTGGTGTCCAACATCG	Sequencing
Metarhizium_ifT1_F6	CAACCCACGAGGATGAAGG	Sequencing
Metarhizium_ifT1_F7	CTTGGTTCGCAAGGGAGAGA	Sequencing
Metarhizium_ifT1_F8	GCAATCTACACCGCAGAAGTTG	Sequencing
Metarhizium_ifT1_F9	GAATGTCCTGCTCGTCAGA	PCR and sequencing
Metarhizium_ifT1_F10	GCATCTGCTCTTGAGGTCAGA	Sequencing
Metarhizium_ifT1_F11	CAACGAATTCCATGATCGCGAG	Sequencing
Metarhizium_ifT1_F12	GTCAAGATCAAGAGCGAGACTCG	Sequencing
Metarhizium_ifT1_F13	CCATTCTTGATCTTCTGGAGAAGC	Sequencing
Metarhizium_ifT1_F14	CAAGACTGCTCTTTGCACTCTC	Sequencing
Metarhizium_ifT1_F15	GCTCATGTTCATGCTCTTCACTG	Sequencing
Metarhizium_ifT1_F16	CTGGCAGTACATGGAGGACTA	Sequencing
Metarhizium_ifT1_R1	CGAGCGTGTAATTTACTCCT	PCR and sequencing
Metarhizium_ifT1_R2	CACATGAAGATCCAGAATCGTGG	Sequencing
Metarhizium_ifT1_R3	CTTGAAGAAGATGAAGCCGATGAAC	Sequencing
Metarhizium_ifT1_R4	GATGGTGCAAAGAACAGCTTGAC	Sequencing
Metarhizium_ifT1_R5	CCATAAGCGCGGTTAGAGTAC	Sequencing
Metarhizium_ifT1_R6	CTCACATTGGCGTATTTGGTGAG	Sequencing
Metarhizium_ifT1_R7	CAATTGGCCGTTGTGCGTAAAG	Sequencing
Metarhizium_ifT1_R8	GTTCTTCCAGGCAGTAGCAAAC	PCR and sequencing
Metarhizium_ifT1_R9	CATAGCCATAACAACATCGCGTAG	Sequencing
Metarhizium_ifT1_R10	TGCAAGGCCAATTGGCTTTTC	Sequencing
Metarhizium_ifT1_R11	CTGCAGAGGTTCGCGAGTAG	Sequencing
Metarhizium_ifT1_R12	CTCAAGCGGACAAGGCGAT	Sequencing
Metarhizium_ifT1_R13	CATTTTGCTCAAGGCCCCTT	Sequencing
Metarhizium_ifT1_R14	GACTGCGCATCGCTTCAAGAT	Sequencing
Metarhizium_ifT1_R15	CATTCGTCCTTCTCCTCTCATCC	Sequencing
Metarhizium_ifT1_R16	GTACCAGCATTCGCCAAACTG	Sequencing

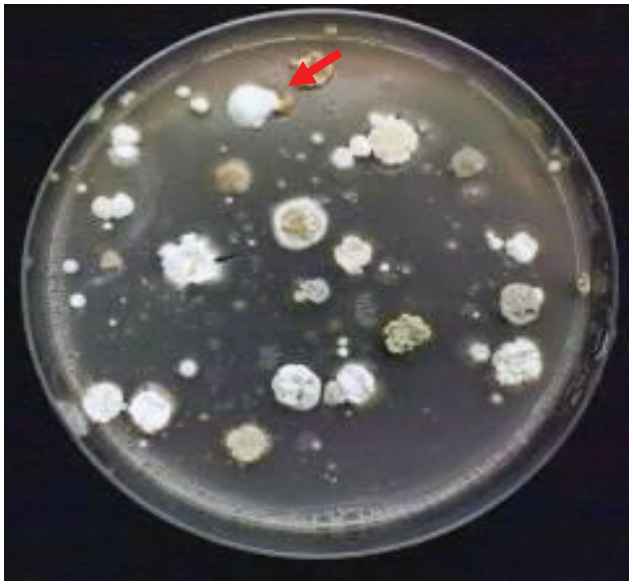

Figure 11. Thermotolerant derived from *M. anisopliae* wild-type conidia irradiated by ion beams (300 Gy) on SDA incubated at 38°C. Mutants should be indicated by arrows.

Table 16. Origin of thermotolerant mutants developed by ion-beam or gamma-ray irradiation.

Wild-type	**Mutant**	**Irradiation (dose, Gy)**
AcMa5	AcMa5-ib	Ion-beam (300)
	AcMa5-gr-1	Gamma-ray (100)
	AcMa5-gr-2	Gamma-ray (100)
	AcMa5-gr-3	Gamma-ray (1000)
PaMa02	PaMa02-ib	Ion-beam (100)

Table 17. Colony diameter (mm, mean ± SD) of wild-type and mutant isolates of *M. anisopliae* on SDA at different temperatures.

Isolate	Temperature (°C)									
	15	20	25	30	33	35	36	37	38	39
AcMa5 (wild-type)	4.3 ± 0.6	15.0 ± 0.7	23.7 ± 0.3	22.0 ± 0.7	16.1 ± 0.2	5.4 ± 0.5	2.1 ± 1.0	0	0	0
AcMa5-ib	2.7 ± 0.6 *	19.3 ± 1.3	34.2 ± 0.5 *	27.9 ± 1.0 *	18.9 ± 0.7 *	12.2 ± 0.4 *	4.9 ± 0.2 *	1.0 ± 0.0 *	0.7 ± 0.6	0
AcMa5-gr-1	4.1 ± 0.2	19.1 ± 1.0	24.7 ± 0.7	22.3 ± 0.3	18.7 ± 0.7 *	11.0 ± 0.0 *	6.8 ± 0.2 *	3.2 ± 0.4 *	1.7 ± 0.6 *	0
AcMa5-gr-2	3.4 ± 0.5	18.7 ± 0.0 *	26.3 ± 0.6 *	25.2 ± 0.4 *	16.0 ± 1.5	10.8 ± 0.2 *	6.1 ± 0.8 *	3.1 ± 0.2 *	2.3 ± 0.9 *	0
AcMa5-gr-3	2.9 ± 0.7	18.6 ± 0.5 *	27.1 ± 0.2 *	25.5 ± 0.4 *	19.1 ± 0.2 *	11.7 ± 1.2 *	5.6 ± 0.2 *	2.9 ± 0.5 *	2.3 ± 0.6 *	0
PaMa02 (wild-type)	9.9 ± 1.0	19.1 ± 0.2	32.7 ± 1.2	26.0 ± 1.0	15.7 ± 0.6	3.0 ± 0.0	0	0	0	0
PaMa02-ib	8.7 ± 0.6	14.8 ± 0.2 *	23.0 ± 0.0 *	23.0 ± 1.0	15.3 ± 0.7	6.6 ± 1.0 *	2.6 ± 0.5 *	1.1 ± 0.2 *	1.0 ± 0.0 *	0

* indicates significant difference in colony diameter between wild-type and each mutant isolates ($P < 0.05$, Mann-Whitney U test).

Table 18. Characteristic of thermotolerant mutants of *M. anisopliae* (number, size of conidia, colony colour, conidia colour).

Isolate	Number of conidia $\times 10^{7}$ [a)] (ml^{-1})	Size of conidia (µm) [a)]		Colony colour (the presence of conidia)				Conidia colour			
		Length	Width	35°C	36°C	37°C	38°C	35°C	36°C	37°C	38°C
AcMa5	1.96 ± 0.09 bc	5.97 ± 0.81 ab	2.51 ± 0.46 bc	Yellow (-)	Yellow (-)	NG	NG	-	-	-	-
AcMa5-gr-1	3.67 ± 0.75 a	5.70 ± 0.89 bc	2.35 ± 0.59 c	Green (+)	Pale green (-)	Yellow (-)	Pale Yellow (-)	Green		-	-
AcMa5-gr-2	2.57 ± 0.28 b	6.06 ± 0.9 a	2.31 ± 0.59 bc	Green (+)	Pale green (-)	Yellow (-)	Pale Yellow (-)	Green	-	-	-
AcMa5-ib	4.15 ± 0.16 a	5.92 ± 0.70 ab	2.46 ± 0.44 bc	Green (+)	Pale green (-)	Yellow (-)	Pale Yellow (-)	Green	-	-	-
AcMa5-gr-3	2.33 ± 0.07 b	5.97 ± 0.81 ab	2.53 ± 0.43 ab	Green (+)	Pale green (-)	Yellow (-)	Pale Yellow (-)	Green	-	-	-
PaMa02	1.30 ± 0.21 c	5.53 ± 0.82 c	2.48 ± 0.7 bc	Yellow (-)	NG	NG	NG	-	-	-	-
PaMa02-ib	1.42 ± 0.07 c	5.89 ± 0.68 ab	2.68 ± 0.51 a	Yellow (-)	Yellow (-)	Pale Yellow (-)	Pale Yellow (-)	-	-	-	-

[a)] at 25°C for 10 days

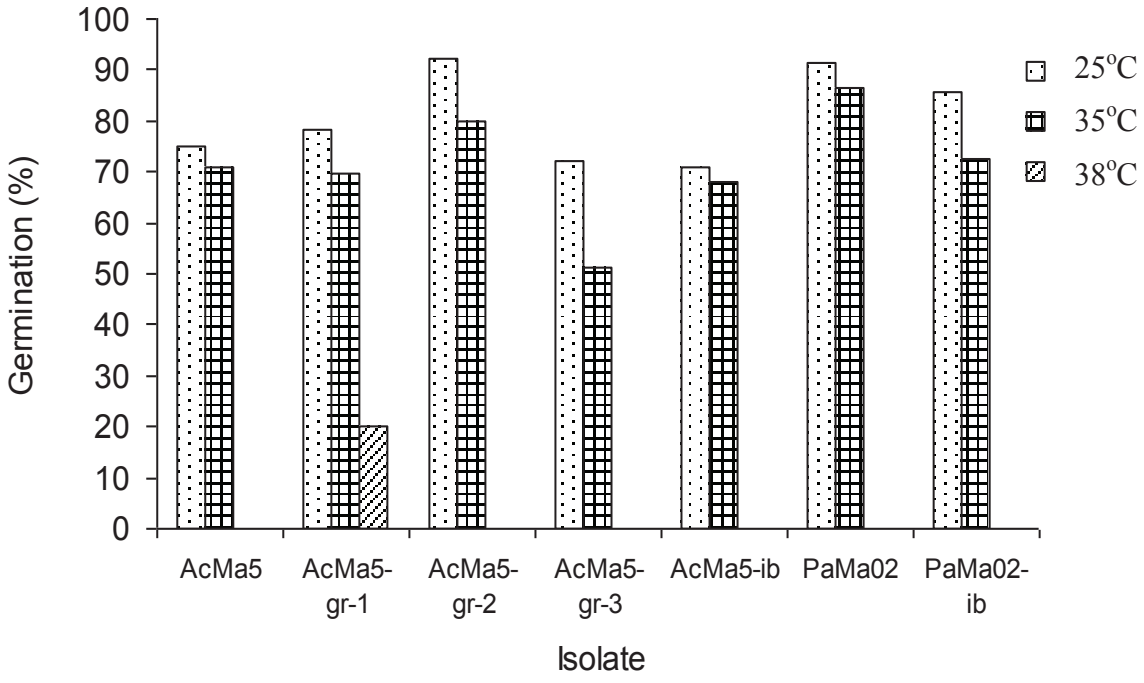

Figure 12. Germination of wild-type and the mutants isolates of *M. anisopliae*.

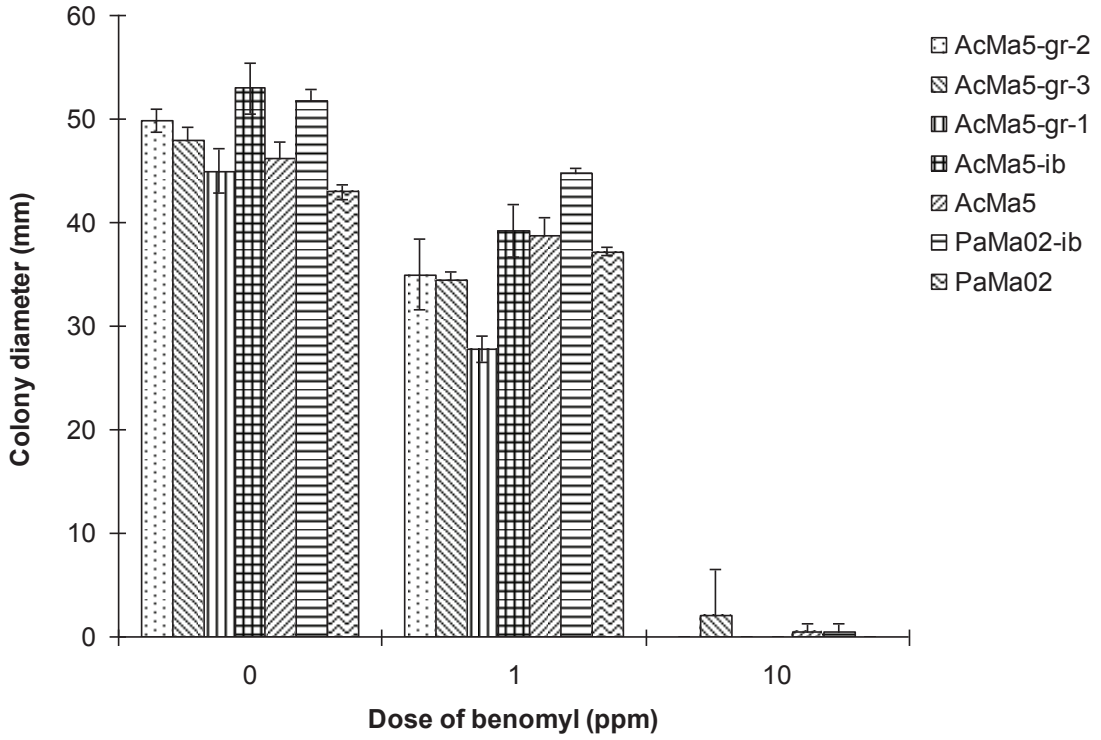

Figure 13. Response of wild-type and mutants isolates of *M. anisopliae* to benomyl. Error bars represent standard errors of five replicates.

Table 19. Percent germination (mean ± SD) of wild-type isolates of *M. anisopliae* and the mutants derived from them after exposure to wet-heat stress of 45°C.

	Hours of exposure to wet-heat stress			
Isolate	0	0.5	1	3
AcMa5 (wild type)	92.3 ± 0.4 a	18.7 ± 3.5 a	0 a	0
AcMa5-ib	93.0 ± 1.8 a	66.1 ± 6.9 d	19.3 ± 1.8 c	0
AcMa5-gr-1	94.3 ± 2.3 a	22.9 ± 3.2 ab	7.5 ± 3.2 b	0
AcMa5-gr-2	96.0 ± 1.3 a	45.9 ± 7.4 c	5.6 ± 1.4 b	0
AcMa5-gr-3	92.4 ± 1.9 a	35.0 ± 5.5 bc	8.8 ± 2.7 b	0
PaMa02 (wild type)	91.6 ± 3.0	16.0 ± 2.3	0	0
PaMa02-ib	91.0 ± 0.5	12.2 ± 2.0	0	0

In AcMa5 and the mutants derived from it, means followed by different letters in each column are significantly different from each other ($P < 0.05$, Tukey HSD test). There was no significant difference between PaMa02 and the mutant derived from it ($P > 0.05$, Student *t*-test).

Table 20. Percent germination (mean ± SD) of wild-type isolates of *M. anisopliae* and the mutants derived from them after exposure to dry-heat stress of 60°C.

Isolate	Hours of exposure to wet-heat stress			
	0	0.5	1	3
AcMa5 (wild type)	91.18 a	0 c	0	0
AcMa5-ib	90.44 a	81.25 a	0	0
AcMa5-gr-1	91.04 a	0 c	0	0
AcMa5-gr-2	92.00 a	74.97 a	0	0
AcMa5-gr-3	90.73 a	29.88 b	0	0
PaMa02 (wild type)	90.40 a	80.67 a	0	0
PaMa02-ib	90.38 a	0 c	0	0

Tukey HSD test ($P = 0.05$)
The number in the same coloumn followed by the same letter is not significantly different.

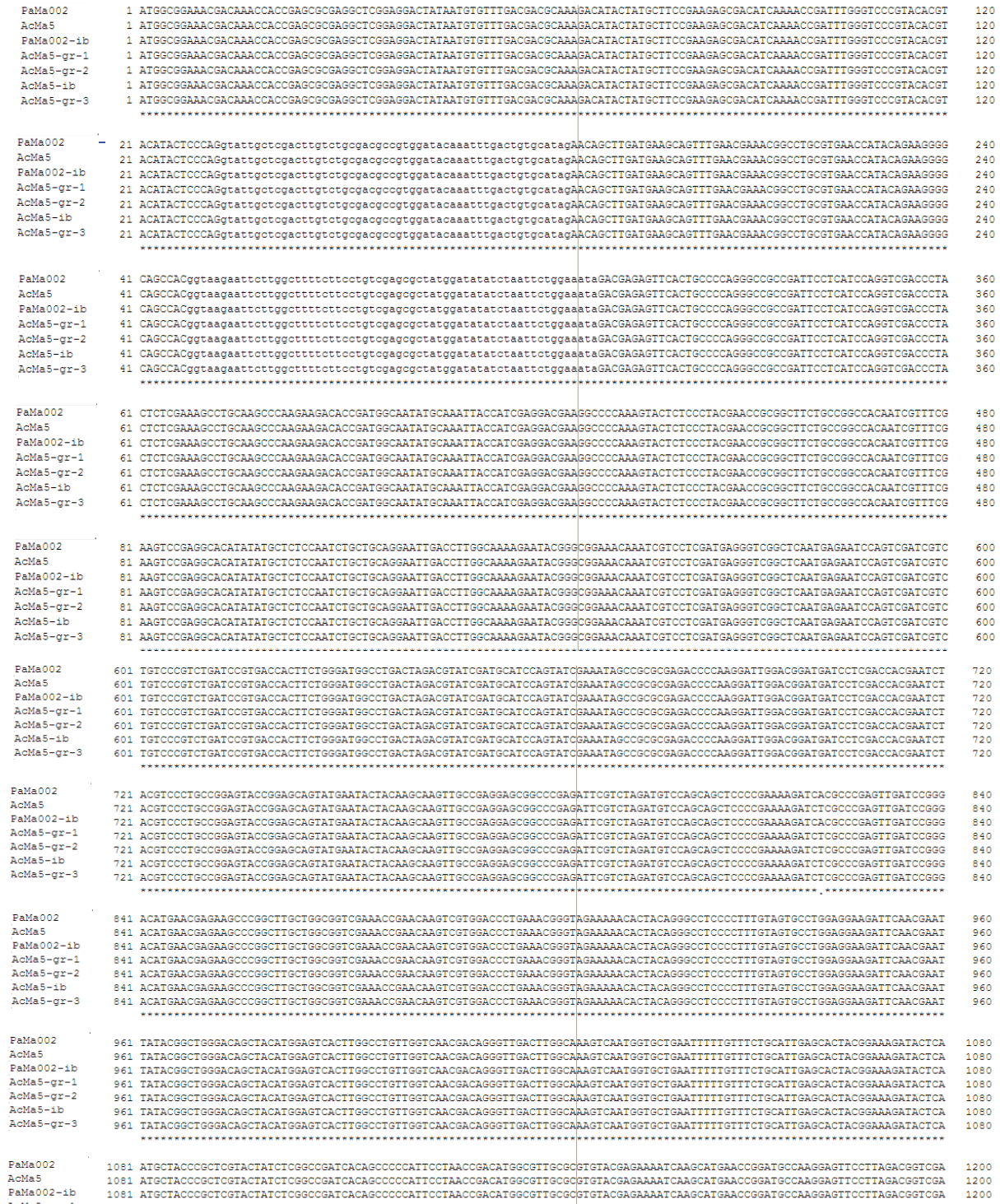

Figure 14. Sequence of *Ntl* locus of the wild-type and mutants isolates of *M. anisopliae*.

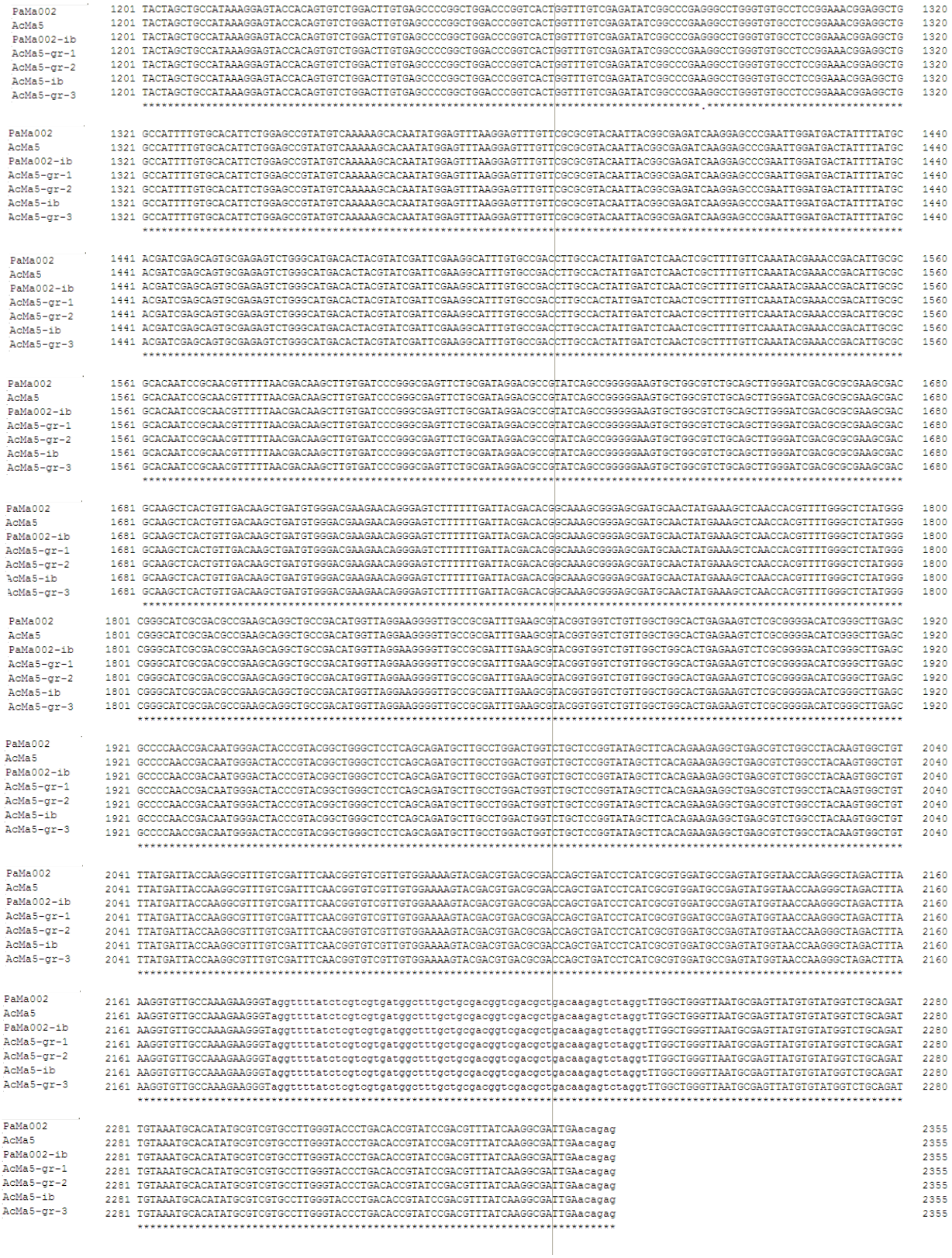

Figure 14. Continued

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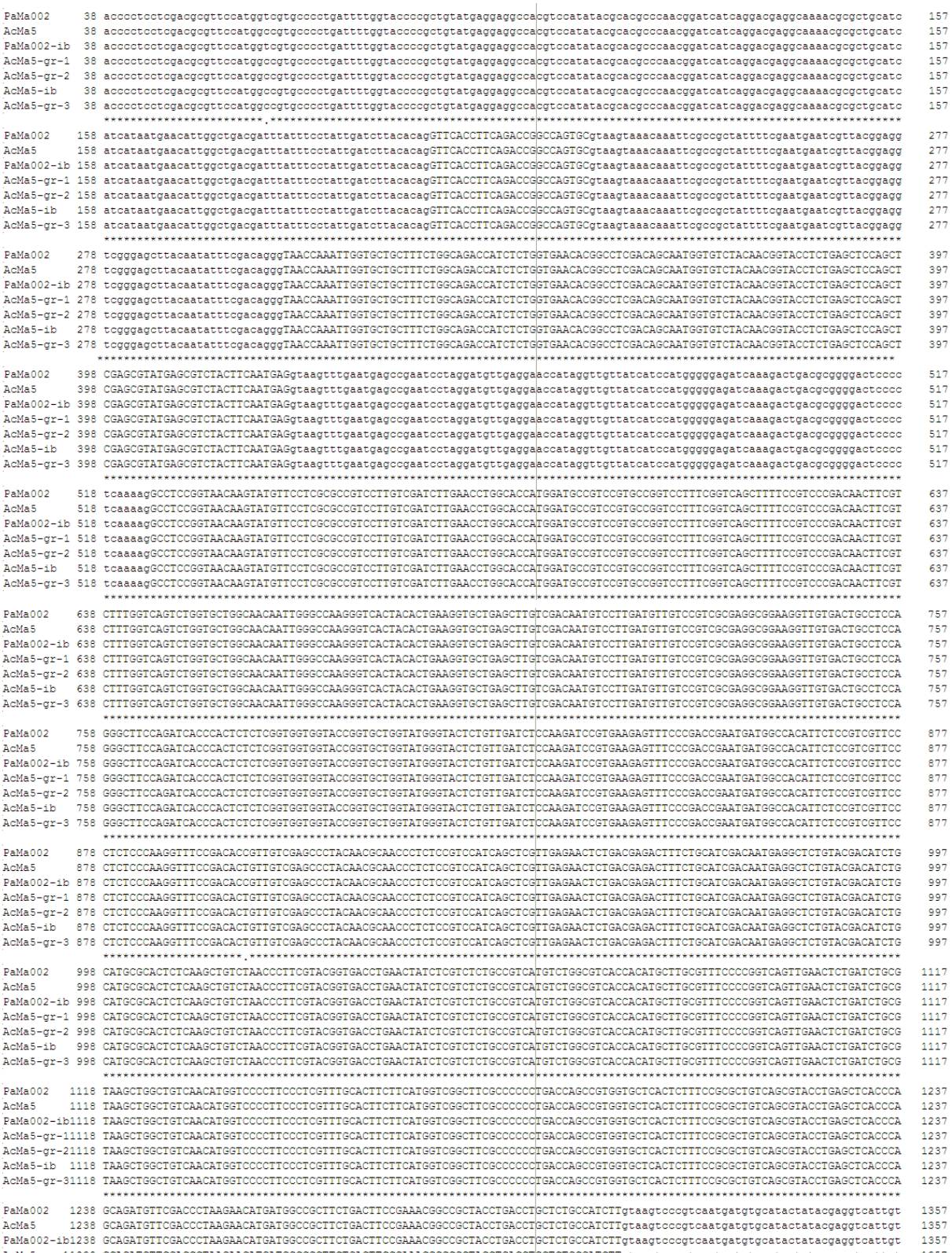

Figure 15. Sequence of *β-tubulin* locus of the wild-type and mutants isolates of *M. anisopliae*.

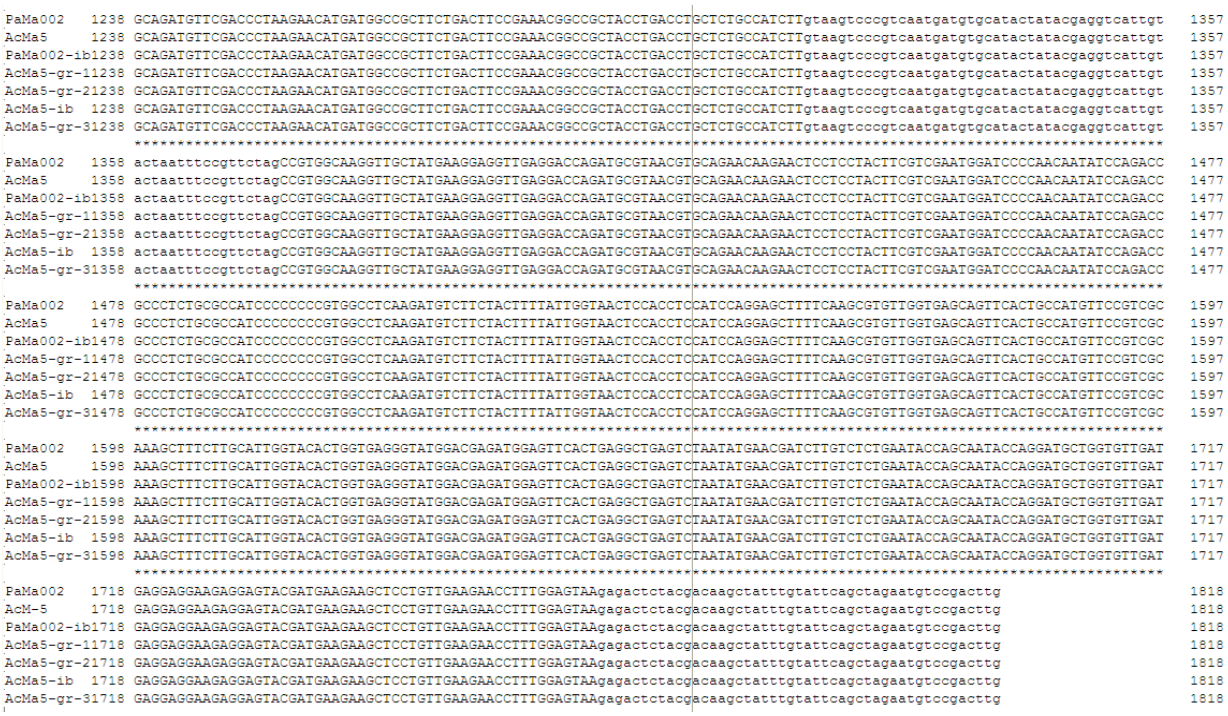

Figure 15. Continued.

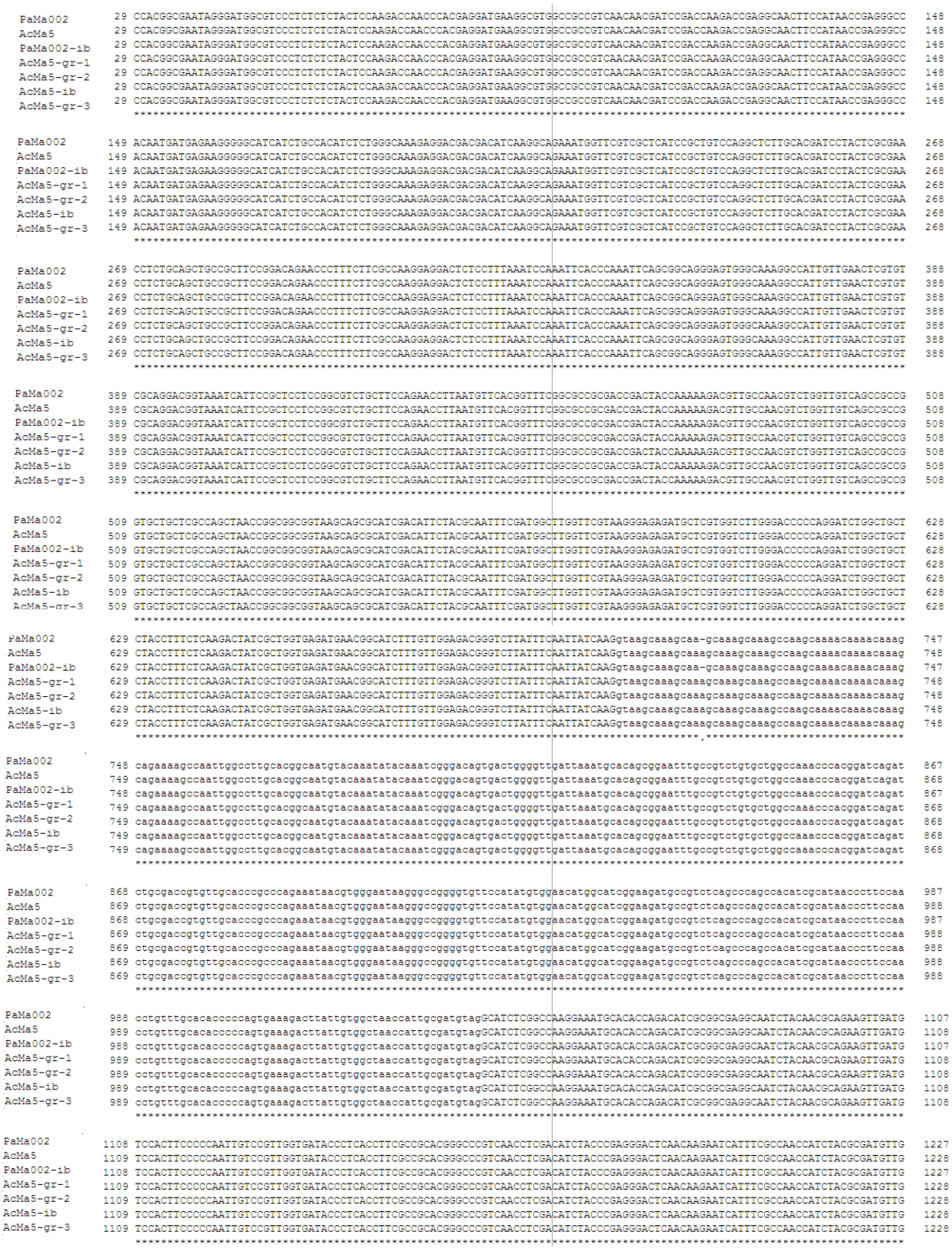

Figure 16. Sequence of *ifT1* locus of the wild-type and mutants isolates of *M. anisopliae*.

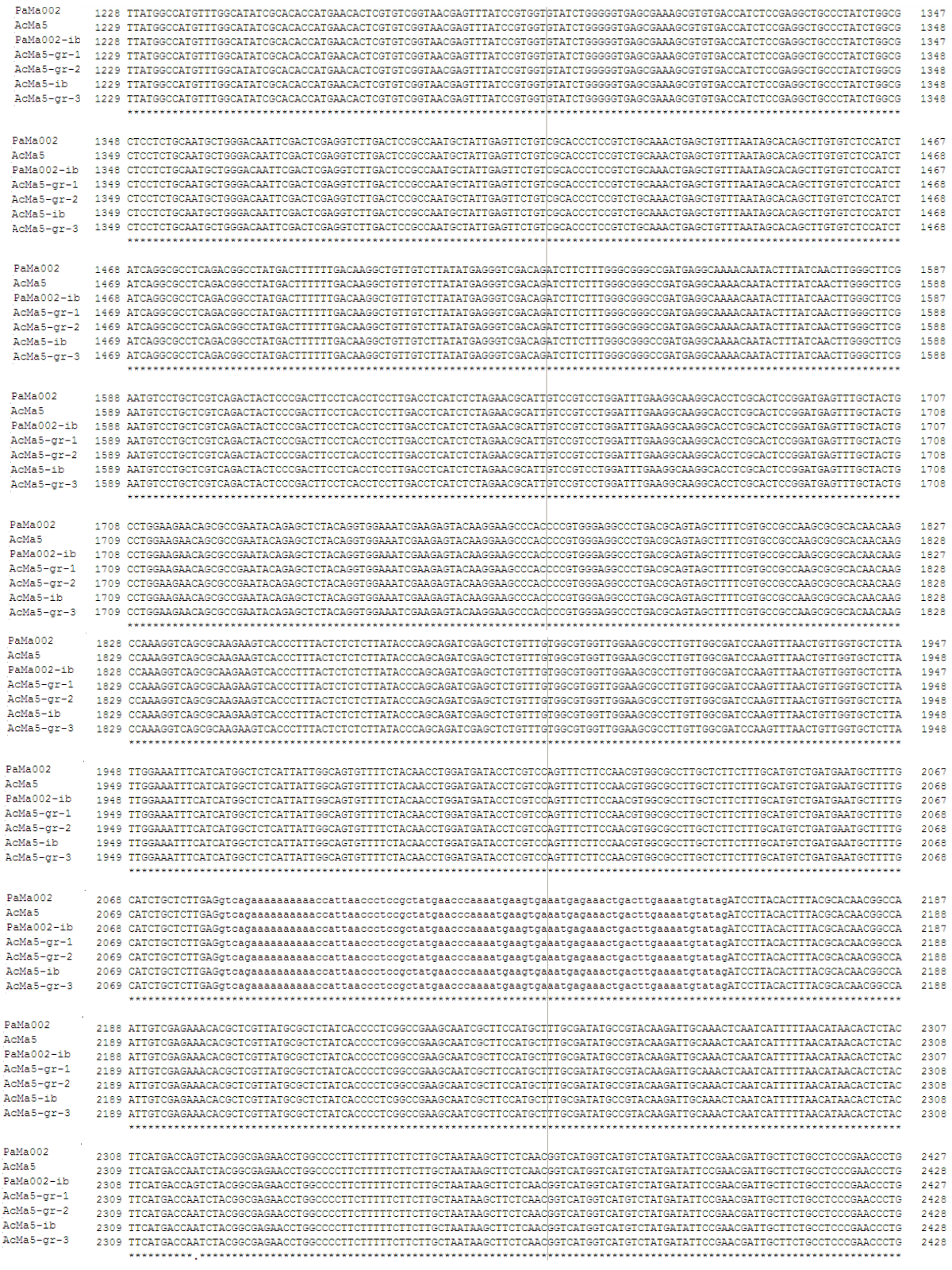

Figure 16. Continued.

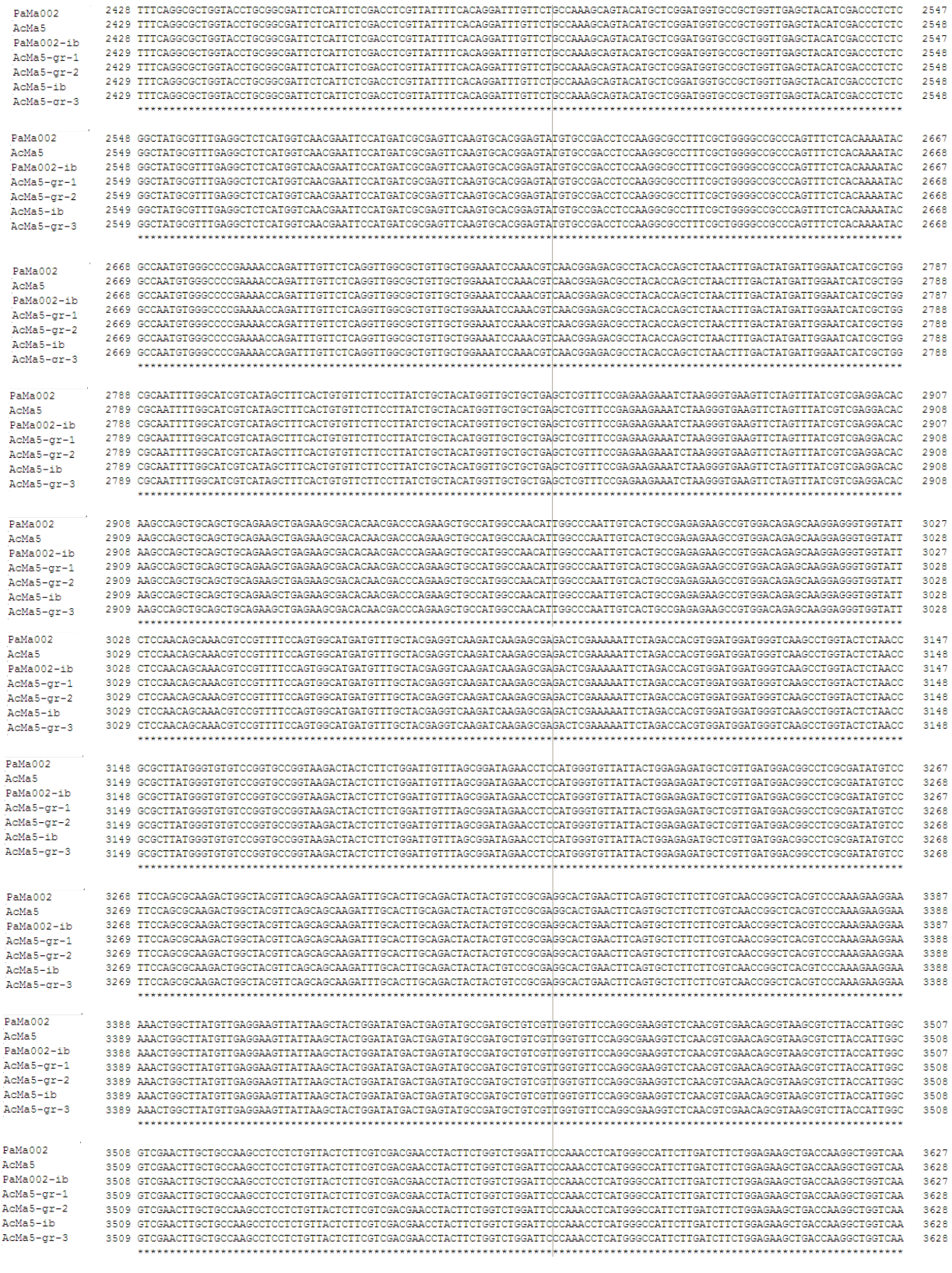

Figure 16. Continued.

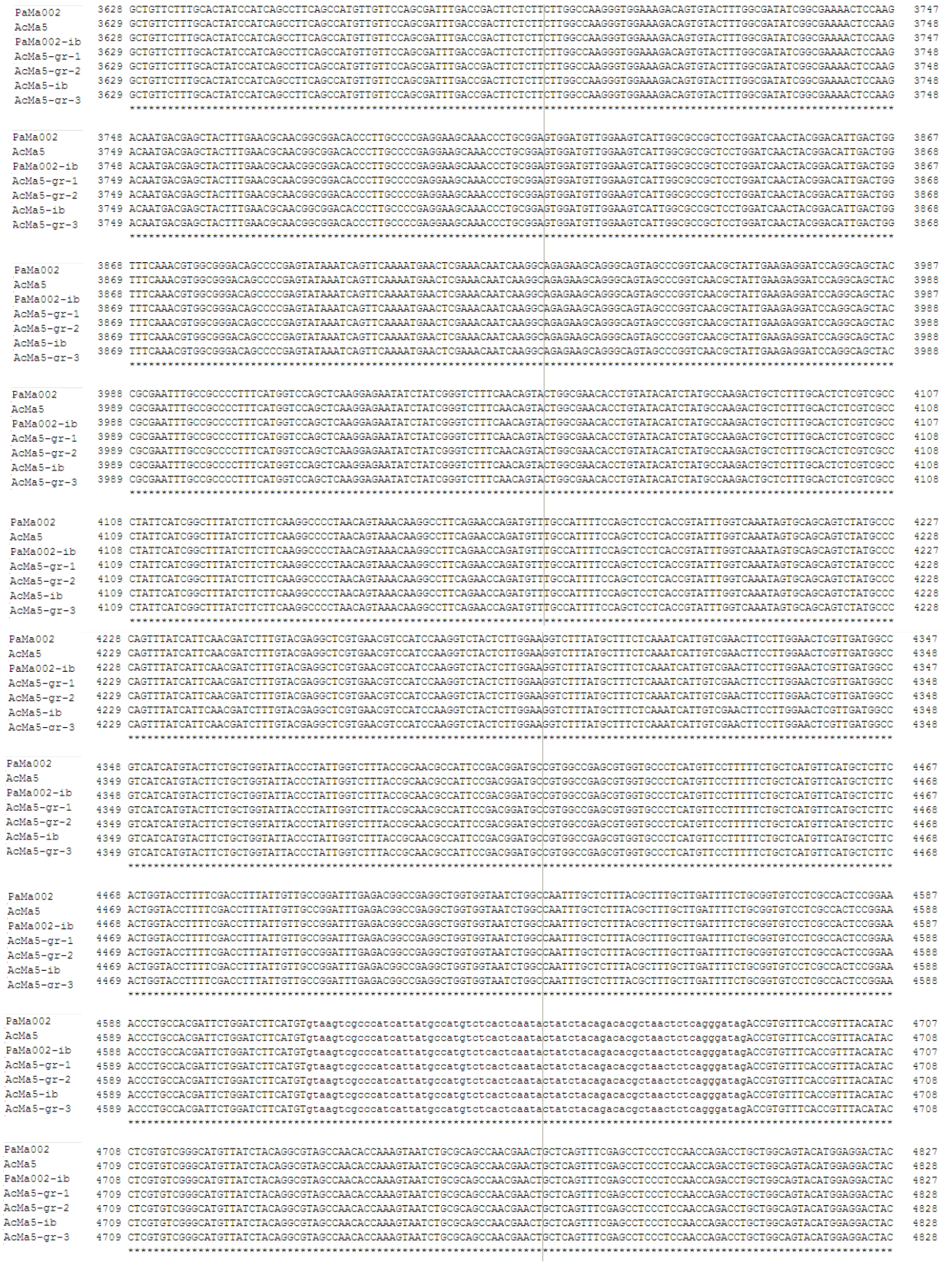

Figure 16. Continued.

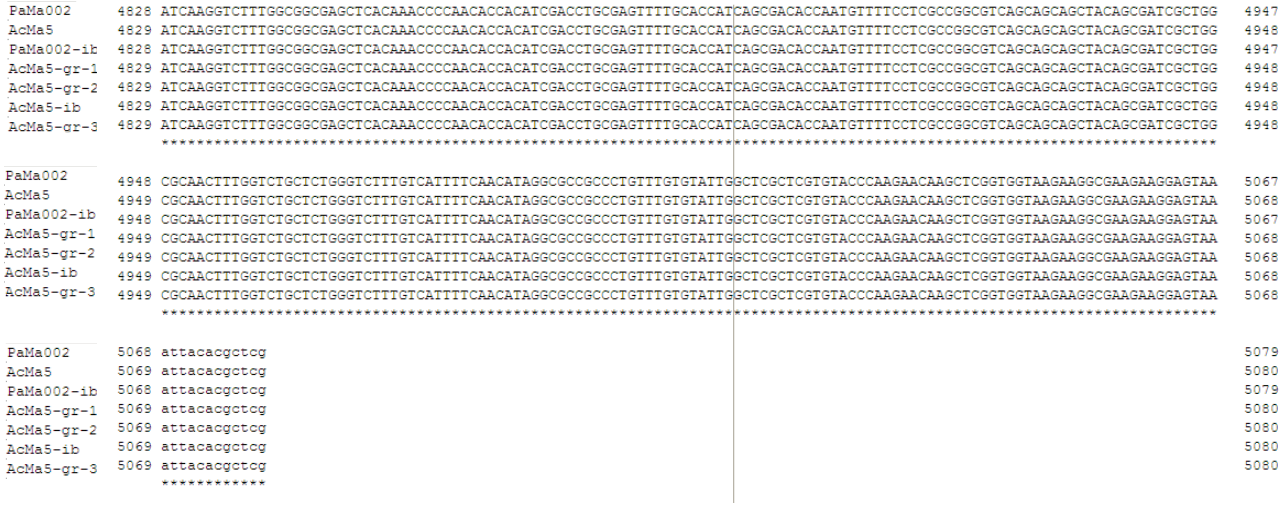

Figure 16. Continued.

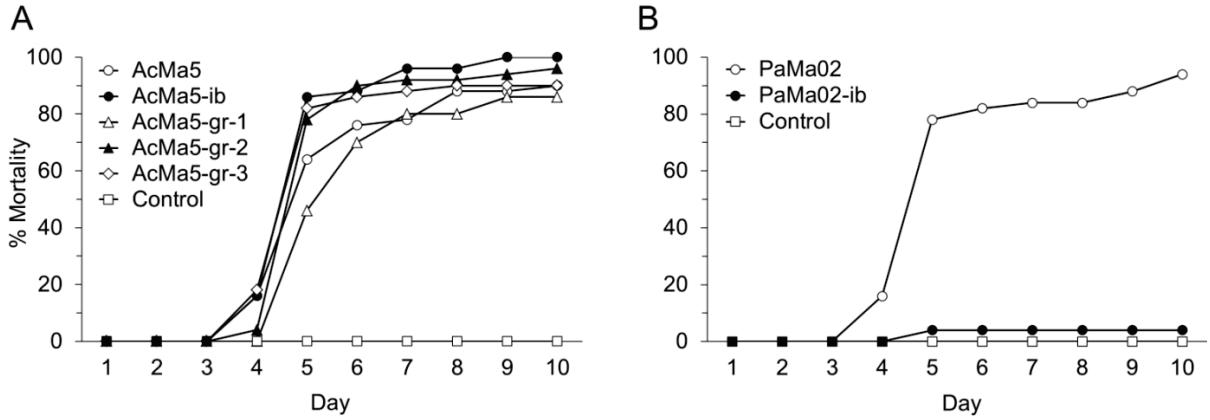

Figure 17. Virulence of wild-type and mutant isolates of *M. anisopliae* against rice weevil adults at 25°C. A. Wild-type isolate AcMa5 and the mutants derived from it. B. Wild-type isolate PaMa02 and the mutant derived from it.

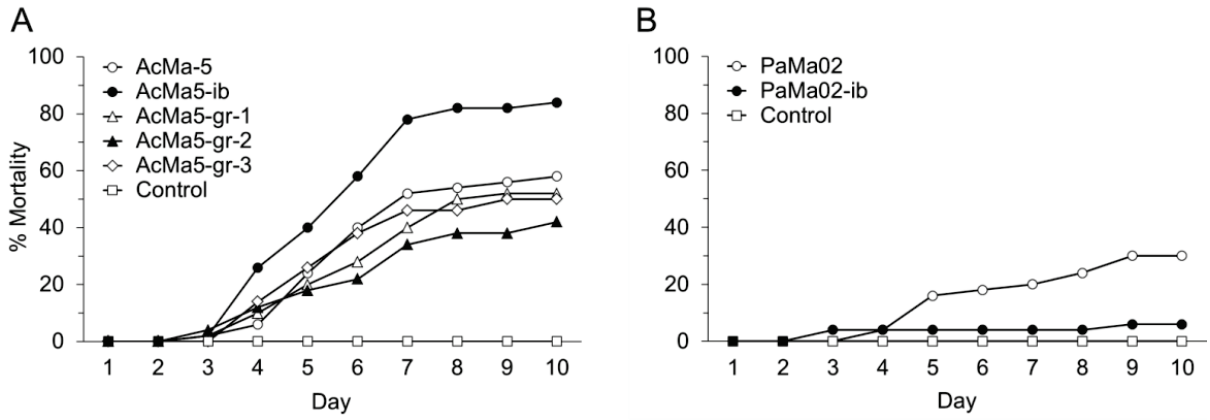

Figure 18. Virulence of wild-type and mutant isolates of *M. anisopliae* against rice weevil adults at 30°C. A. Wild-type isolate AcMa5 and the mutants derived from it. B. Wild-type isolate PaMa02 and the mutant derived from it.

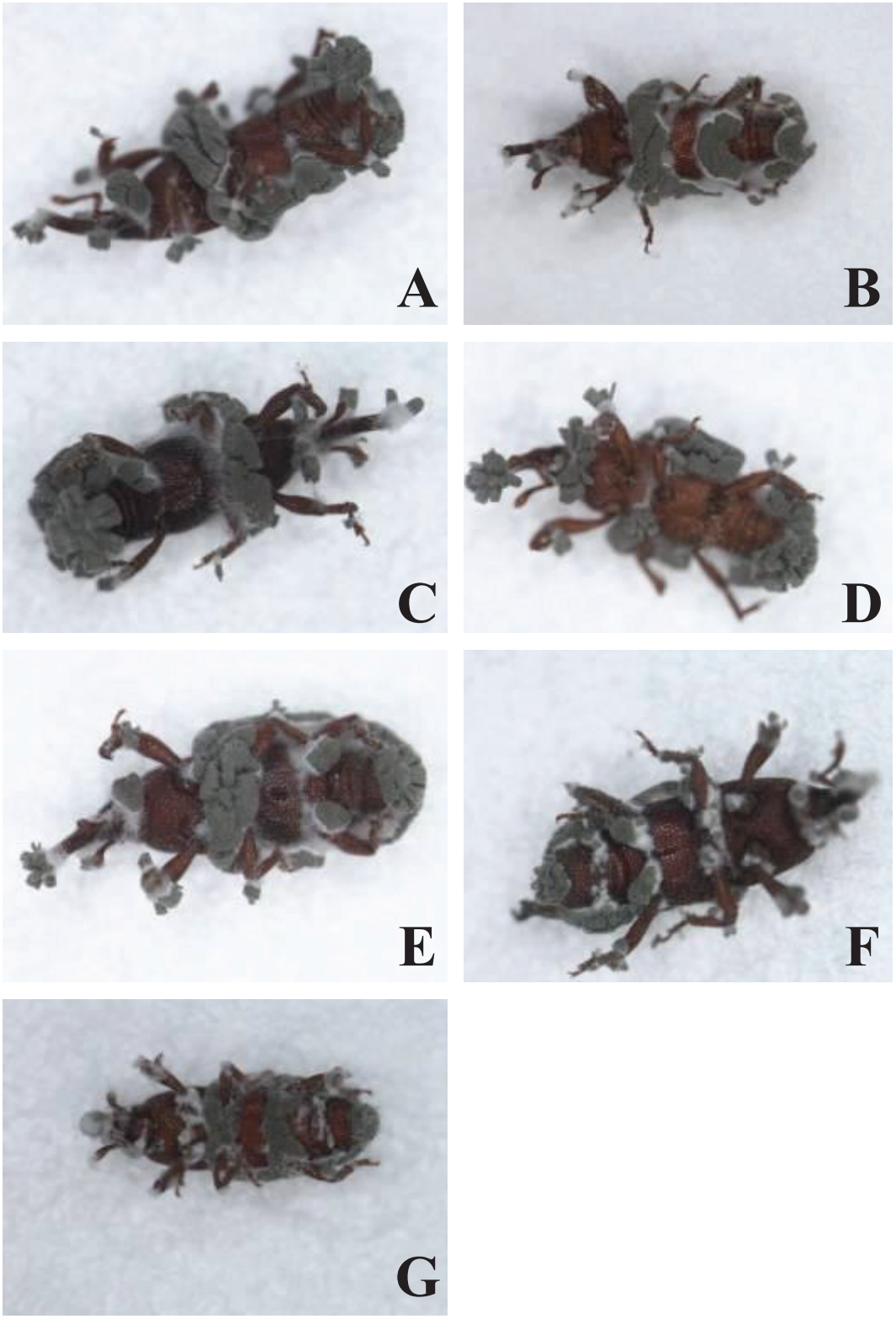

Figure 19. The rice weevil, *S. zeamais*, infected by *M. anisopliae*. A, AcMa5; B, AcMa5-gr-1; C, AcMa5-gr-2; D, AcMa5-gr-3; E, AcMa5-ib; F, PaMa02; G, PaMa02-ib.

CHAPTER 5

GENERAL DISCUSSION

Entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea*, have been widely used as biological control agents which are also frequently used in IPM programs combined with other control methods such as chemicals (Shah and Pell, 2003). However, they are susceptible to fungicides applied for control of plant diseases (Butt et al., 2001; Clark et al., 1982; D'Alessandro et al., 2011; Loria et al., 1983; Pell et al., 2010; Saito, 1984). Generally, the risk of fungicides on entomopathogenic fungi are commonly avoided by making intervals between the applications of each agent (Gardner et al., 1984; Bruck, 2009), however, these intervals is impractical because time consuming reduces the effectiveness of both agents in the field. Heat stress is another problem faced by entomopathogenic fungi (Castrillo et al., 2005; Zimmermann, 2007a, b, 2008). For example, in the temperatures more than 35^{o}C cause severe negative effect on some traits of entomopathogenic fungi, such as conidial germination and persistence, mycelial growth, sporulation, and/or infection to the host insect (Arthurs and Thomas, 2001; Cabanillas and Jones, 2009; Darbro et al., 2011; Dimbi et al., 2004; Fargues et al., 1997; Inglis et al., 1997; Li and Feng, 2009; Thomas and Jenkins, 1997; Vidal et al., 1997), which reduces their efficacies in the field.

In tropical countries, pesticides for controlling pest insects and diseases have been frequently applied where entomopathogenic fungi may be negatively affected. For example, in Indonesia, insecticides and fungicides were highly used mainly in the central

of vegetable production areas, such as in Giham, Sumatera, Indonesia (Barral et al., 2012). In the ornamental production areas, they are also highly used. Chemical residue on crops and in environment has caused serious problems in Indonesia. The high temperatures, which reach 35^{o}C or above in tropical areas, also negatively affect to entomopathogenic fungi (Yasukawa et al., 2009), which may decrease effectiveness of entomopathogenic fungi. Also elevated temperatures in glasshouses possibly result in decreased effectiveness of entomopathogenic fungi.

In order to overcome problems from fungicides and heat stress, many scientist have tried to develop mutants tolerant to fungicides or high temperatures in entomopathogenic fungi. Fungicide-tolerant mutants have been developed by several ways, such as selection on chemically amended media (Shapiro-Ilan et al., 2002, 2011; Butters et al., 2003), by transformation (Pfeifer and Khachatourians, 1992; Inglis et al., 1999; Bernier et al., 1989; Valadares-Inglis and Inglis, 1997), UV (Kim et al., 2005) or by exposure to mutagenic agents such as $NaNO_2$ (Zou et al., 2006; Song et al., 2011). Thermotolerant mutants have also been developed; thermotolerant *M. anisopliae* mutants were developed using UV-B irradiation (Rangel et al., 2006) and continuous culture method (de Crecy at al., 1999).

In this study, six benomyl-tolerance mutants of *I. fumosorosea* (Ib-34, Ib-421, Gr-5, Gr-22, GrIb-8, and GrIb-9), two benomyl-tolerant mutants of *B. bassiana* (BB22 and BB24) and five thermotolerant mutants of *M. anisopliae* (AcMa5-ib, AcMa5-gr-1, AcMa5-gr-2, AcMa5-gr-3, and PaMa02-ib) were obtained using ion-beam and/or gamma-ray radiations. Ion-beam and gamma-ray radiations are applied for breeding of crops and microorganisms (Zengliang, 2006; Anuntalabhocai et al., 2011; Piri et al., 2011). In this study, ion beams and gamma rays were used to improve the traits of

entomopathogenic fungi. This study demonstrated that the methods were potentially useful tools for inducing beneficial mutations in entomopathogenic fungi as biological control agents.

Five benomyl-tolerance mutants (Ib-34, Ib-421, Gr-5, Gr-22, and GrIb-8) of *I. fumosorosea* enhanced their tolerance which were more than 2000-fold than those observed in the wild-type isolate. The tolerance levels were higher than that of ones previously reported. For example, carbendazim-resistant mutants reported by Song et al. (2011) exhibited 830-fold more resistance than the wild-type isolate, which were still less than the five mutants obtained in this study. Meanwhile, two benomyl-tolerance mutants of *B. bassiana* enhanced their tolerance which were more than 500-fold compare to the wild-type isolate. Enhanced benomyl-tolerance in the mutants developed in this study may be sufficient to avoid the negative effects of benomyl applications in the field (500 mg l^{-1} is the recommended application rate).

Benzimidazole fungicides, such as benomyl, are negatively cross-resistant to N-phenylcarbamate fungicides, such as diethofencarb (Fujimura et al., 1992a; Ziogas & Girgis, 1993; Leroux et al., 1999). In this study, the benomyl-tolerant isolates of *I. fumosorosea* showed similar or little more sensitivity to diethofencarb than the wild-type isolate. On the other hand, the mutants enhanced tolerance to thiophanate-methyl, another benzimidazole chemical, which may be resulted from cross-tolerance among benzimidazoles (Davidson et al., 2006; Keinath and Zitter, 1998). The mutants also showed enhanced tolerance to to chlorothalonil, triflumizole (only BB22) and iprodione (only BB24) compared with the wild-type isolate. This exhibits multiple mechanisms conferring tolerance to several different fungicides including benzimidazoles. All mutants should be screened for undesirable mutations that may

have occurred alongside the desirable mutation conferring benomyl tolerance.

Mutation sites in the *β-tubulin* locus of benzimidazole-tolerant plant pathogenic fungi have been reported (e.g. Albertini et al., 1999; Baraldi et al., 2003; Chen et al., 2009; Koenraadt et al., 1992; McKay and Cooke, 1997; Qiu et al., 2011), however, many studies have stressed on the replacement of the amino acid at position 198 and/or 200 (e.g. Davidson et al., 2006; Fujimura et al., 1992b; Hollomon et al., 1998; Koenraadt and Jones, 1993; Kongtragoul et al., 2011; Ma et al., 2003; Schmidt et al., 2006; Yarden and Katan, 1993). Replacement of amino acid at position 198 resulted in high tolerance to benzimidazole fungicides in the phytopathogenic fungi as reported by Ma et al. (2003) and Koenraadt et al. (1992). In entomopathogenic fungus *B. bassiana*, enhanced tolerance to benzimidazole chemicals was obtained when mutation was occurred at position 198 with replacement of glutamate with lysine (E198K), glycine (E198G), or valine (E198V) (Butters et al., 2003; Zou et al., 2006). In this study, replacement of glutamate with alanin at position 198 (E198A) was observed in the mutants derived from *B. bassiana*, confirming that mutation at this position is very important. In contrast, no mutation in the *β-tubulin* locus was observed in mutants derived from *I. fumosorosea*. The benomyl-tolerance mutants developed by Song et al. (2011) also did not possess any mutations at the *β-tubulin* locus. These findings indicate that there are different mechanisms in mutation of benomyl-tolerance between *B. bassiana* and *I. fumosorosea*.

All the thermotolerant mutants resulted different responses to temperatures. Three mutants (AcMa5-ib, AcMa5-gr-2 and AcMa5-gr-3) derived from wild-type isolate AcMa5 grew significantly faster than the wild-type isolate and another mutant AcMa5-gr-1 (Table 17). In contrast, a mutant PaMa02-ib generated from wild-type isolate PaMa02 grew significantly slower than the wild-type isolate (Table 17). Thus, the

traits resulted by mutagenesis derived from the same irradiation method could not be predicted. Different traits of the mutants were also observed in their upper thermal limit. The wild-type isolate AcMa5 and all its mutants exhibited 37 and 39^{o}C, respectively, indicating that these mutants enhanced thermotolerance by 2^{o}C (Table 17). Similarly, a mutant PaMa02-ib exhibited upper thermal limit of 39^{o}C which was higher than that of the wild-type isolate PaMa02 by 3^{o}C (Table 17). Thermotolerant mutants of *M. anisopliae* were also developed by Crecy et al. (1999). Using continuous culture method, they produced thermotolerant mutants with upper thermal limit at 38^{o}C. The mutants developed in this study showed 1^{o}C higher in the upper thermal limit than the mutant developed by Crecy et al. (1999).

This study found no mutation in the sequence of three genes, neutral trehalase locus (*Ntl*) (Song et al., 2011), *β-tubulin* locus (Zou et al., 2006), and the ABC transporter locus (*ifT1*) (Leng et al., 2011), which are possibly responsible for thermotolerance of entomopathogenic fungi. This finding shows that these three genes have no association with thermotolerance in *M. anisopliae*.

Ion beams are one of high–Liner Energy Transfer (LET) radiation which shows higher relative biological effectiveness of lethality, cell inactivation, and higher rate of DNA double-strand breaks compared to the low LET irradiation such as gamma rays and X rays (Blakely, 1992; Lett, 1992; Tanaka et al., 2010; Murai et al., 2013; Yamaguchi, 2013). Among the three mentioned ionizing irradiations, ion beams produce the highest mutation frequency followed by gamma rays and X rays (Tanaka et al., 2010; Ahloowali et al., 2004). However, there were little reports on use of ionizing radiations to develop mutants of entomopathogenic fungi, though exposure of the entomopathogenic fungus *Cordyceps militalis* to ion beams successfully generated a

mutant isolate capable of enhanced production of cordycepin, a medical adenosine analogue (Das et al., 2008, 2010).

In this study, ion-beam and gamma-ray irradiations are showed as useful tools for improving traits, such as enhancement of fungicide-resistance in *I. fumosorosea* or fungicide-tolerance in *B. bassiana*, and thermotolerance in *M. anisopliae*. These methods potentially produce novel characteristics in entomopathogenic fungi because they cause a high mutation frequency and a broad mutation spectrum and create point mutations in genes (Matuo et al., 2006; Zengliang, 2006; Tanaka et al., 2010; Toyoshima et al., 2012). Point mutation is useful to analyse the genes associating with individual traits in entomopathogenic fungi. For example, we clarified that mutation at codon 198 in *β-tubulin* gene associated with benomyltolerance in *B. bassiana*. By understanding point mutation of the genes within entomopathogenic fungi, the genes associating with other traits, such as sporulation ability and virulence, may be specified, including the gene associated with benomyl-tolerance in *I. fumosorosea* and thermotolerance in *M. anisopliae.*

In this study, three kinds of insects, the tobacco whitefly (*Bemisia tabaci*), the onion thrips (*Thrips tabaci*) and the rice weevil (*Sitophilus zeamais*), were used in virulence tests for the mutants. Both the tobacco whitefly and the onion thrips are important pests because of their wide host-range and chemical resistance (Oliveira et al., 2001; Perring, 2001; Reitz et al., 2011; Sakimura, 1947; Cortês et al., 1998; Hsu et al., 2010; Wijkamp et al., 1995). Therefore, in this study, the two insect species were used for virulence tests in *I. fumosorosea* and *B. bassiana* mutants, respectively. Meanwhile, the rice weevil was not a target of *M. anisopliae* mutants because the insect is in stored grain pests (Tefera et al., 2011; Caneppela et al., 2003). However, a wild-type of *M.*

anisopliae was originally isolated from a scarab (Coleoptera), so that the rice weevil was used for virulence tests in *M. anisopliae* mutants. Further studies are needed to examine virulence of each fungal mutant using other important pests belonging to order Lepidoptera, Coleoptera, Homoptera, Thysanoptera, etc.

As described in each chapter, undesirable mutations were observed for conidial production, germination ability, and virulence in the mutants generated by ion beams or gamma rays. Therefore, the resulting mutants should be carefully evaluated for unpredictable negative effects (Shapiro-Ilan et al., 2011) before field application.

CONCLUSION

Ion-beam radiation is a useful tool for improving traits, such as enhancement of fungicide resistant in *I. fumosorosea* or fungicide tolerance, in *B. bassiana* and thermotolerance on *M. anisopliae*. This radiation method potentially produced novel characteristics in entomopathogenic fungi. However, because mutant entomopathogenic fungi can show unpredictable negative trait changes, the resulting mutants should be carefully evaluated for other traits.

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