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Novel point mutations in β-tubulin gene for carbendazim resistance maintaining nematode pathogenicity of *Paecilomyces lilacinus*

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Abstract The application of fungicides is so critical, especially in greenhouses, to avoid fungal infections. Carbendazim, an inhibitor of tubulin biosynthesis, is the most widely known broad-spectrum benzimidazole fungicide. The application of carbendazim affects other beneficial fungi as well. *Paecilomyces lilacinus* 36-1 (*Pl*36-1) is a beneficial fungus used for biological control, and the most effective biocontrol agents of nematode eggs. The *Pl*36-1 is sensitive to carbendazim (0.3 μg/ml). There is a general consensus that the mechanisms of resistance to carbendazim in the β-tubulin gene have been analyzed in detail. However, no studies were conducted on *P. lilacinus* strains. In the present study, two carbendazim-resistant mutants of *Pl*36-1, P50 and P100, were obtained from UV exposure and tested. The β-tubulin gene fragments were cloned and sequenced in the three strains, *Pl*36-1, P50 and P100. The resistance to carbendazim was developed when amino acid substitutions occurred at β-tubulin gene positions of S145A, T185A and F200Y. The β-tubulin gene was overexpressed in *Pl*36-1 strains. The β-tubulin expression level of the overexpressed mutant (PL3), quantified by qRT-PCR, was increased 4-folds over its normal level in *Pl*36-1. In vitro, the PL3 was resistant to carbendazim with maintaining growth, sporulation and pathogenicity rates. Three-year field trial demonstrated that P100 and PL3 strains exhibited carbendazim resistance combined with high nematode reduction and yield improvement.

Keywords β-tubulin · *Paecilomyces lilacinus* · Carbendazim resistance · Amino acid substitutions · *Meloidogyne incognita*

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Introduction

Nematodes are destructive plant pathogens responsible for yield losses in numerous economic crops. A huge amount of damage is caused by endo-parasitic sedentary nematodes which generate particular feeding sites in plant roots. The most common of these pathogens, root-knot nematodes "RKN" (family: Meloidogynidae) are the major plant parasitic species with economic value (de Almeida Engler et al. 2005). Root-knot nematodes (*Meloidogyne* spp.) are obligatory and substantial parasites of many economic crops (Javed et al. 2006). Controlling root-knot nematodes is of major economic importance to the agricultural industry of China (Huang et al. 2014). *Meloidogyne arenaria*, *M. incognita* and *M. javanica* are the main species responsible for yield losses in vegetable cropping areas (Sikora and Fernandez 2005). These nematodes belonging to this

genus play a crucial role for their important phytosanitary implications often pave the way to emergent, opportunistic or well-known pathogenic fungi on cultivated crops (Bertrand et al. 2000; Dimartino et al. 2011; Gallo et al. 2011; Starr et al. 1989; Vitale et al. 2011, 2014). Recently, the most efficient chemical control products (e.g., methyl bromide) have been restricted due to their animal and human toxicity. Research on biological control agents (BCAs) against root-knot nematodes which have no deep impact on the environment is becoming increasinglyrequested (Yang et al. 2011; Abdelnabby et al. 2011). Many fungi and their metabolites are known to have nematicidal activity against a wide range of plant-parasitic nematodes including RKNs (Kiewnick and Sikora 2006; Hallman et al. 2009). In the last two decades, many efforts yielded an adequate number of bioformulations containing several BCAs. Among the well-accepted commercial products is the fungus *Paecilomyces lilacinus* (Lamovsek et al. 2013).

Paecilomyces lilacinus (Thom) Samson, a soil-inhabiting and egg-parasitic fungus, is one of the most effective opportunistic parasites of nematode eggs (Khan et al. 2006). There are promising results with the use of *P. lilacinus* 251 (PL251) as a biological control agent against various plant-parasitic nematodes (Anastasiadis et al. 2008; Yang et al. 2011; Crow 2013). PL251 strain reduces infestation with *M. incognita* by nearly 66 % (Kiewnick and Sikora 2006). Commercial products of *P. lilacinus* are marketed in Europe, Central America and North Africa (Wilson and Jackson 2013). According to the common agricultural practices, *P. lilacinus* applications are combined with fungicide treatments to prevent secondary and associate infections caused by soilborne fungi (Anastasiadis et al. 2008). *Paecilomyces lilacinus* 36-1 was isolated from *M. incognita* in our laboratory and showed effective control of root-knot nematode as well as soybean cyst nematode diseases. The isolated *P. lilacinus* 36-1 was sensitive to the fungicide carbendazim applied at a rate of 0.3 μg/ml. Its efficacy on nematode control has been reduced when carbendazim was applied to control fungal diseases under greenhouse and field conditions.

Resistance to carbendazim fungicide has been detected with several species of fungi in laboratory and field studies (Davidse and Ishii 1995; Brent and Hollomon 2007). It is well known that the basic resistance mechanism of fungal species to fungicides is due to a modification at the target-site of fungicidal action (Brent and Hollomon 1998). The interrelationships of carbendazim resistance with point mutations of the β-tubulin gene have been analyzed in details (Verhey and Gaetig 2007; Hammond et al. 2008; Cheng et al. 2009). Many studies have shown that the mechanism of action for carbendazim fungicides is binding to β-tubulin and preventing α-tubulin assembly (Fujimura et al. 1992; Cools et al. 2011; Cools and Fraaije 2012). High and long persistence of resistance is characterized in the field after interrupted applications of the carbendazim fungicide due to β-tubulin amino acid substitutions E198A, E198V, E198K and F200Y (Georgopoulos and Skylakakis 1986; Yarden and Katan 1993; Leroux et al. 2002; Ma and Michailides 2005). Polymerase chain reaction (PCR) diagnostic assays can detect the E198A resistance mutations in *Botrytis cinerea* isolates (Luck and Gillings 1995; Banno et al. 2008). However, the molecular mechanisms of carbendazim-resistance in *P. lilacinus* are poorly detected and understood. Thus, the aim of this paper was focused on analysis of the carbendazim target sites of the β-tubulin gene from resistant transformants to improve carbendazim-resistance with maintaining nematodes virulence of *P. lilacinus*.

Materials and methods

Strains and culture conditions

A wild strain of *P. lilacinus* 36-1 (*Pl36-1*), susceptible to 0.3 μg/ml of carbendazim, was isolated from eggs of *M. incognita* in our laboratory. The strain was cultured on potato dextrose agar (PDA) medium at 28 °C for 7 days. For long-term storage, it was maintained in glass tubes on PDA medium at 4 °C in darkness with one transfer every 6 months. *Sclerotinia sclerotiorum* A (SA) strain stored in our laboratory was cultured on PDA medium at 22 °C for 4 days.

Development of UV-induced carbendazim-resistant mutants of *P. lilacinus* 36-1

The UV-induced carbendazim-resistant mutants were developed according to the procedure of Tanaka et al. (1988). Ten ml aliquots of *Paecilomyces lilacinus* 36-1 conidial suspension (1×10^4 conidia/ml) containing Tween 20 (0.02 % *v*/*v*) was poured into a petri plate and exposed to UV irradiation (254 nm Phillips TUV

25 W/h) at a distance of 15 cm for 0.5, 1, 1.5 and 2 min. The conidia were incubated in the dark for 8 h to prevent photo-reactivation. Aliquots of 100 μl of treated conidia (1×10^4 conidia/ml) with mutagens were plated separately on PDA amended with carbendazim at the rate of 1 μg/ml. The petri plates were incubated at 28 C for 3 days. The growing mutant strains were transferred to PDA medium containing carbendazim solution at concentrations of 1, 5, 10, 50, 60, 100 and 150 μg/ml. Survival rates were determined from the ratio of colonies which grew on fungicide amended media and number of conidia inoculated.

Stability test for fungicide resistance of tested mutants

Carbendazim resistant mutants were subcultured on fungicide-free PDA medium for 10 generations. Consequently, the mutants were transferred to the respective fungicide "1 μg/ml" amended PDA medium. Stability rate was determined from the ratio of survived colonies which grew on the fungicide amended medium and number of colonies inoculated. The carbendazim resistant mutant strains obtained by UV-irradiation were designated.

Extraction of DNA and RNA, and cDNA synthesis

P. lilacinus cultures were grown on PDA at 28 °C for 7 days. Mycelia were peeled and ground under liquid nitrogen. Genomic DNA was extracted using 2 % CTAB buffer. Total RNA was isolated using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA. Genomic DNA was removed using TransScript TM II RT/RI Enzyme Mix (Transgen).

Sequence analysis of *β*-tubulin gene

The specific degenerate primers bctubF (5′-ACAACC GCCAACATGC GTGAGATTGTGA-3′) and bctubR (5′-CTCCATCTCGTCCATTCCTTTCGCCTG -3′) were designed based on the cited sequences of *N. haematococca* mpVI 77-13-4, *A. fumigatus* Af293, *T. stipitatus* ATCC10500, *A. niger* CBS531.88 and *G. zeae* PH-1, in the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/). These primers were used to amplify full-length sequences of gDNA in 50 μl of PCR reaction system containing 5 μl of 10×LA PCR BufferII (Mg^{2+} Plus) (TaKaRa), 8 μl of 2.5 mM deoxyribonucleoside triphosphate (dNTPs) (TaKaRa), 1 μl of each primer (20 μM of each bctubF and bctubR), 0.5 μl of 5 U/μl LATaq (TaKaRa) and 32.5 μl of sterilized distilled water (ddH_2O). Genomic DNA (2 μl) from *Pl*36-1 and P100 were applied as templates. The PCR cycling conditions were 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 63.5 °C for 1 min, and 72 °C for 2 min; then finally 10 min extension at 72 °C. The PCR products of the expected size were ligated to pMD18-T vector (TaKaRa) and at least 10 independent clones from each strain were sequenced in both directions and analyzed. Sequence data were analyzed using DNAStar software (DNAstar, Madison, USA).

Phylogenetic characterization

The sequences of the PCR products were compared to known *β*-tubulin gene sequences in the GenBank database by multiple sequence alignment using ClustalX 1.83 and GeneDoc. Phylogenetic tree construction was performed using the neighbor-joining method with MEGA 5.1.3.

3D homology model of developed *β*-tubulin

The change of *β*-tubulin 3-D appearance may affect fungicide binding ability leading to carbendazim-resistance of *P. lilacinus* mutants. Three-dimensional and surface structures of the *β*-tubulin gene protein were successfully predicted and constructed using Pymol software (http://www.pymol.org/). The constructed surface structure of P100 mutant was compared with that of wild strain (*Pl*36-1), to determine the amino acid substitutions and their effect on the surface structure appearance.

Expression vector construction

For further studies, six new vectors p1, p2, p3, p4, p5 and p6 were reconstructed based on pEGAD-CBX vector containing carboxin resistance gene CBX (Fig. 1). In the six applied vectors, the *β*-tubulin gene would substitute bar gene as a selectable marker. The amino acid residues, S145A and F200Y, of *β*-tubulin gene were substituted in p1 vector, T185A and F200Y in p2 vector, S145A, T185A and F200Y in p3 vector, S145A in p4 vector, while T185A in p5 vector, and S145A and T185A in p6 vector. The applied vectors were

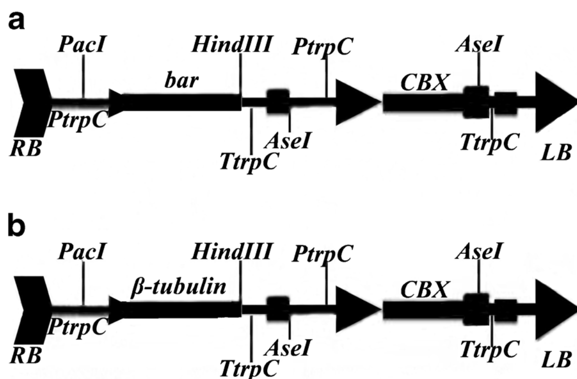

Fig. 1 Construction of the expression vectors. **a** T-DNA of the binary vector *pEGAD- CBX* containing two selectable markers coding for resistance genes (*bar* and *CBX*). **b** T-DNA of the binary vectors P1, P2, P3, P4, P5 and P6 containing two selectable markers coding for resistance genes (*β*-tubulin and *CBX*). The *β*-tubulin gene had two amino acid residues S145A and F200Y were substituted in p1 vector, T185A and F200Y were substituted in p2 vector, and S145A, T185A and F200Y were substituted in p3 vector, S145A in p4 vector, while T185A in p5 vector, and S145A and T185A in p6 vector

respectively transferred to *P. lilacinus* and *S. sclerotiorum* A strains using agrobacterium-mediated (ATMT).

Expression level of *β*-tubulin

The applied primers for quantitative RT-PCR (qRT-PCR) were bctubF/R for *β*-tubulin gene and actF/R (actF:5'- ATCCACATCACCACTTGCAA; actR:5'- TGCTCGGAGATCGACATTTG) for the actin gene. All data were normalized to actin gene expression. Relative changes in gene expression levels were analyzed. qRT-PCR data from three biological replicates were used to calculate the mean and standard deviation (SD) in the expression level of each gene.

Virulence test of *P. lilacinus*

Laboratory tests

The pathogenicity of *P. lilacinus* 36-1 and the transformant strains were examined against *M. incognita* by calculating the rate of egg parasitism and larval mortality. A total of 0.5 mL of conidial suspension (10^6 CFU/ml) was mixed with 0.5 ml eggs or juvenile suspension of *M. incognita* (100 eggs/ 0.5 ml or 100 juveniles/ 0.5 ml). The control was prepared by mixing 0.5 ml of H_2O with 0.5 ml of eggs or juveniles suspension. All treatments were incubated at 28 °C. Three replications were applied for each treatment. The egg parasitism ratio and nematode mortality rates were recorded for each treatment at 48-h time intervals. The experiment lasted 6 days.

Field experiments

Three *P. lilacinus* strains P36-1, P100, and PL3 were selected for further tests under field conditions. The field experiment was conducted in a controlled greenhouse with a serious nematode infection, (Huazhong Agricultural University, Wuhan, China). The experiments were conducted at three successive seasons: December 2012 to February 2013 on cucumber, November 2013 to January 2014 on tomatoes and March 2014 to June 2014 on cucumber. During these periods, the average of temperature was 25.8±2 °C, while relative humidity recorded 56±3 %. The experiment adopted a randomized complete block design with five replicate plots (5× 2 m) per treatment. Plots within blocks were separated by 1 m-wide buffers and blocks were separated by 2 m-wide buffers. After 4 weeks of crop growth, 2 l spore suspension (107 CFU/ml) of each *P. lilacinus* treatment was uniformly sprayed at each plot. One week later, 2 l of carbendazim (100 μg/m) was sprayed on each fungal treated plot. The plot free of spores suspension and carbendazim is a negative control (CK-), while the plot which received carbendazim and free of fungal treatment is control (CK). At the end of growth season, soil and plant roots were collected. Nematodes were extracted from sub-samples of 100 g soil with a modified Baerman Funnel method as described by Hooper (1984). Additionally, root gall index (mean root samples of 20) was determined on a scale of 0 to 5 based on the percentage of the root system with galls (Hussey and Janssen 2002), where 0=no galling; 1=trace infection with a few small galls; 2=≤ 25 % roots galled; 3=26 to 50 %; 4=51 to 75 %; and 5 = >75 % roots galled.

Statistical analysis

Statistical analysis was carried out using SPSS 12.0.1. The data were expressed as mean±SD. Groups were compared by Duncan's Multiple Range Test to identify

significance of difference between groups. P value< 0.05 was considered statistically significant.

Results

Induction of UV mutation of *P. lilacinus* 36-1

UV method has been applied to produce a number of laboratory mutants. Seven hundreds of mutants were produced and screened on PDA amended with carbendazim at concentration of 1 μg/ml. Resistant colonies were picked up and stored in test tube slants amended with 1 μg/ml carbendazim. Data reported in Tables 1 and 2 show the obtained colonies by UV irradiation and the survival rates with different concentration levels of carbendazim. The tested strains which retained resistant after 10 generations were considered while selecting the suitable mutants for further analysis. The P100 mutant was chosen for further studies according to its high resistance and stability. The β-tubulin gene of P100 was compared to P50 and the sensitive strain *Pl*36-1.

Sequencing β-tubulin gene and phylogenetic characterization

Nucleotide sequences of *Pl*36-1, P50 and P100 were obtained by PCR amplification and sequencing. The full length of the β-tubulin gene of *P. lilacinus* 36-1was 1624 bp, cDNA was 1206 bp, encoding 402 amino acids. The full-length sequence and cDNA of the β-tubulin gene of P100 were the same like *Pl* 36-1 (Fig. 2). Amino Acids Sequence Comparison of β- tubulin gene with common plant pathogenic fungi using the blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated high homology at amino acid level ranging from 95.3 to 99.2 % and containing five exons and four introns (Table 3).

The described bioassay data indicated modifications in the target-site responsible for resistance to carbendazim. The amino-acid positions of amplified β-tubulin gene varied within the range from codon145 to 200. Compared to the β-tubulin gene of *Pl*36-1, the amino acid residues of S145A, T185A and F200Y were replaced in P100 strain while only one amino acid residue “F200Y” was replaced with the strain of P50. On the other hand, both mutants did not produce any change in the amino acid residue of E198 (Fig. 2). Based on the obtained translation of nucleotide sequences, the amino-acids and their site replacements of the β-tubulin gene of the three strains are shown in Table 4. The phylogenetic trees showed a very close relationship (88 %) between *P. lilacinus*, *Penicillium mameffei* and *Acremonium chrysogenumgi* (Fig. 3).

Table 1 Pl36-1 mutations induced *by* ultraviolet light

Exposure time (min)	Mutation		% of survival
	No. of mutants	No. of survived colonies	
0.5	648	149	23
1	593	190	32
1.5	413	331	80
2	647	97	15

Spore concentration: 1×10^4, Incubation period: 3 days, No. of colonies survived and % of survival were calculated according to stability test experiment

Table 2 Survival rates of mutant strains under different concentrations of carbendazim

Concentration of carbendazim (μg /mL)	No. of survived colonies	% of survival
1	500	100
5	492	98.4
10	485	97
50	473	94.6
60	356	71.2
100	320	64
150	10	12.4

Spore concentration: 1×10^4, Incubation period: 5 days

Three-dimensional structures of the β-tubulin gene protein

Modeling of the 3-dimensional and surface structures of the β-tubulin gene protein were successfully predicted and constructed using Pymol software (http://www.pymol.org/). The surface structure of the codons 145, 185 and 200 in P100 strain were changed compared to

Fig. 2 Amino acid alignment of putative β-tubulin protein. Accession numbers: *Acremonium*, P41741.1; *Gibberella*, XM_389706.1; *Verticillium*, ABA61128.1; *Sclerotinia*, XP_001594844.1; *Botryotinia*, XP_001561037.1; *Neurospora*, AAA33617.1; *Colletotrichum*, ELA34262.1. Comparing to the β-tubulin gene of the wild strain *Pl*36-1, the mutants P50 and P100 have a tyrosine residue instead of phenylalanine at position 200, while the P100 mutant has two more points, S145A and T185A

the wild-strain *Pl*36-1. The amino acid residues Serine 145, Threonine 185 and Phenylalanine 200 were replaced with Alanine 145, Alanine 185 and Tyrosine 200, respectively. According to amino acid substitutions in 3 sites, the 3D structure of P100 tubulin has been changed (Fig. 4).

Table 3 Comparison of the β-tubulin gene among different fungal species

Pathogenic fungi	Accession number of nucleotide and amino acid sequence	The number and size of introns	Homology at amino acid level %
Paecilomyces lilacinus[a]	KC415035	4 (198,76,62,56 bp)	/
Sclerotinia sclerotiorum	AY312374	4 (182,8,48,53 bp)	98.9
Gibberella pulicris (tub2)	AF414866, AAN03787 95.78	3 (197,57,48 bp)	97.4
Gibberella fujikuroi	U27303, AAB18275 95.78	4 (179,59,48,49 bp)	99.2
Colletotrichum gloeosporioides	U14138, AAA62875 96.96	6 (184,58,71,56,71,53 bp)	95.3
Botryotinia fukeliana	Z69263, CAA93254 97.66	6 (135,53,69,56,52,56 bp)	95.9
Neurospora crassa	M13630, AAA33617 95.78	6 (240,74,68,65,73,57 bp)	96.1
Rhynchosporium secalis	X81046, CAA56936 96.02	6 (132,55,77,70,52,50 bp)	96.3

Paecilomyces lilacinus[a] : *Paecilomyces lilacinus* 36-1 strain

Table 4 Amino acid mutation points of the β-tubulin gene of *Paecilomyces lilacinus*

Strain	Amino acid at codon			
	145th	185th	198th	200th
P36-1	S	T	E	F
P50	S	T	E	Y
P100	A	A	E	Y

A=alanine; *F*=phenylalanine; *S*=serine; *T*=threonine; *E*=Glutamic acid; *Y*=tyrosine

Fungicide-resistance of transformant strains

Six transformant strains, varied in their carbendazim resistance level, were obtained from the wild strain, *Pl* 36-1. The overexpressed strains (PL1, PL2, PL4, PL5, and PL6) via p1, p2, p4, P5 or p6 vector could not grow in PDA containing 100 μg/ml carbendazim and 400 μg/ml of carboxin, while they could grow in PDA containing 50 μg/ml carbendazim. The transformant strain (PL3), S145A, T185A and F200Y substitutions, could normally grow in PDA containing 100 μg/ml carbendazim. However the substitutions of S145A and/or T185A could not succeed to induce carbendazim-resistant (100 μg/ml) strain, such success was achieved when both substitutions were combined with F200Y. According to the carbendazim-resistant test, PL3 was chosen to be implemented in the next biological applications and expression level quantification. Due to anticipatory cross-resistance relationship between carbendazim and diethofencarb in *P. lilacinus*, diethofencarb-resistance tests for concerned isolates of *P. lilacinus* were conducted. The results exhibited that, *P. lilacinus* 36-1, P50, P100, and PL3 were resistant (70 μg/ml) to diethofencarb. The hyphal growth and conidial germination of all tested strains were not affected by diethofencarb concentrations up to 1.0 μg/ml (Supplementary Fig. 2a and b). According to *Sclerotinia sclerotiorum* A, the transformant strain containing p3 vector (SL3) could normally grow in PDA containing 100 μg/ml carbendazim, while the wild strain was growing in PDA containing no more than 10 μg/ml carbendazim. *S. sclerotiorum* A was used as an example to confirm the feasibility

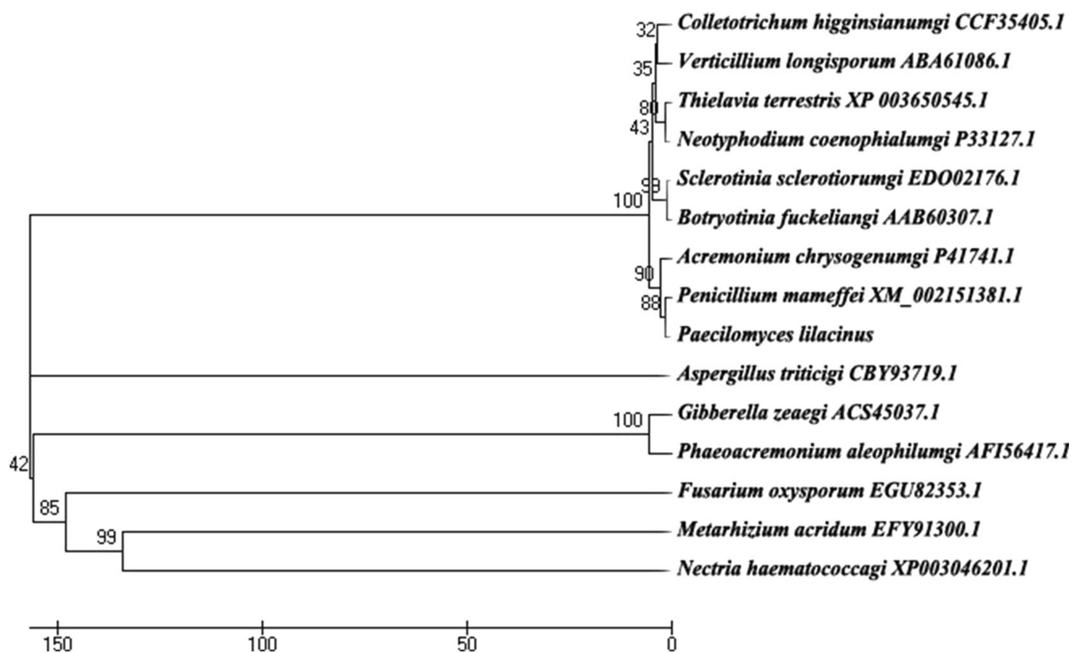

Fig. 3 Phylogenetic trees showing the relationship of *P. lilacinus* 36-1 (*Pl*36-1) to closely related species. The tree was inferred from β-tubulin gene sequence data by the neighbor-joining method. The *scale bar* indicates the estimated number of substitutions per 100 and 50 bases, respectively. Numbers at the nodes indicate the levels of bootstrap support calculated from 1000 trees. Species names are followed by GenBank accession numbers

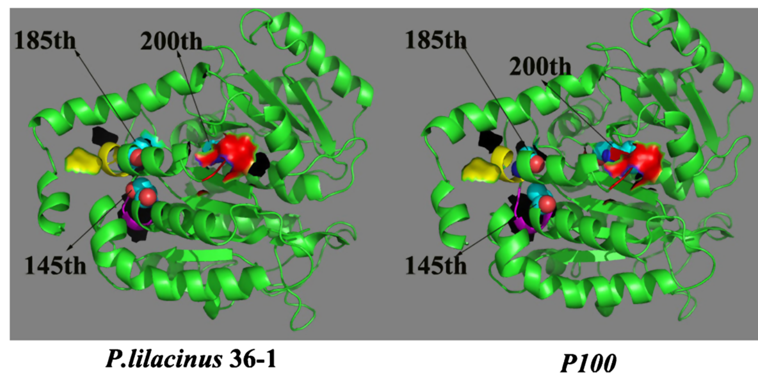

Fig. 4 Modeling of the three-dimensional structures of the β-tubulin gene protein. Two diagrams were constructed with Pymol software. The exposed surface structures of β-tubulin gene I (141–145), II (181–185), and III (196–200) are marked in *magenta*, *yellow*, and *red*, respectively. Spheres structures of β-tubulin gene are marked in cyan

of inserting modified β-tubulin gene with other fungal species.

Expression level of β-tubulin

Expression levels of β-tubulin gene were quantified by qRT-PCR to analyze changes in β-tubulin expression in PL3 and SL3 relative to *Pl*36-1 and *S. sclerotiorum* A, respectively. The quantification level of β-tubulin gene expression in PL3 increased 4-fold over its normal expression level in *Pl*36-1. Moreover, β-tubulin expression level with SL3 increased 4.5-fold over its normal level in *S. sclerotiorum* A (Fig. 5).

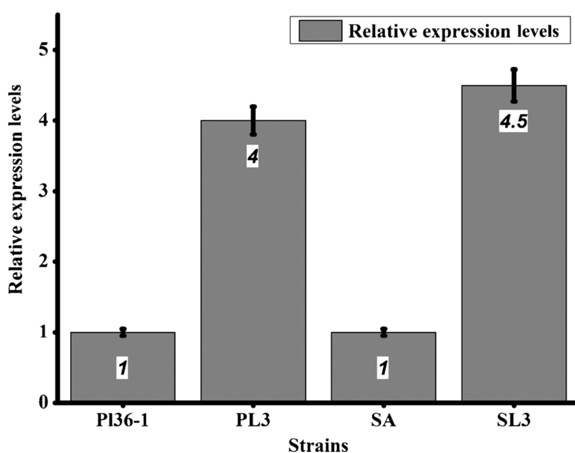

Fig. 5 qRT-PCR analysis of β-tubulin expression. Relative to the expression levels in the parent strains, PL3 increased about 400 % and SL3 increased 450 %. PL3 and SL3 are mutants with β-tubulin gene transformation in *P. lilacinus* and *S. sclerotiorum* strains, respectively. The presented data express the mean±SD of three biological replicates. All comparisons with the relevant parent strain were statistically significant ($P<0.05$)

Fitness characteristics of *P. lilacinus*

Fitness of wild type, mutant and overexpressed strains of *P. lilacinus* were evaluated in vitro to examine the behavior of the transformant strains. The results revealed that the differences in fitness were not significant between all tested strains. The growth rate and sporulation of PL3, β-tubulin transformant strain with p3 vector, recorded 0.60 cm /dish with conidial concentration of 3.81×10^{7} CFU/ dish, while the parent strain *Pl*36-1 achieved growth rate of 0.59 cm/dish with conidial concentration of 3.84×10^{7} CFU/ dish (Table. 5 and Supplementary Fig. 1). The conidia and mycelia of the PL3 strain were identical in shape and size of its parent strain *Pl*36-1. According to the pathogenicity against *Meloidogyne incognita*, the test confirmed that the transformant strain PL3 was still maintaining the pathogenicity level of its parent strain, *Pl*36-1 against eggs and larvae of *M. incognita*. PL3 achieved 63.6 % of egg parasitism and its metabolites killed 68.2 % of the nematode larvae compared to 63.5 and 68.4 % with *Pl*36-1, respectively (Table 5). The previous results provide evidence that the alteration of β-tubulin gene in *P. lilacinus* did not influence the fungal growth, reproduction and virulence against *M. incognita*.

Three-year field experiments were conducted to test the stability and effectiveness of carbendazim-resistance strains at the population level. Data in Table 6 confirmed that the P100 and PL3 strains achieved high performance and persistence in the field throughout each year. Moreover, both strains recorded yield improvement ranging between 20.5 and 24.5 %. No significant ($p>0.05$) differences were detected between the fitness criteria of P100 and PL3 strains. On the contrary, statistically significant ($p<0.05$) changes were detected between both mutants and their parent strain *P. lilacinus*

Table 5 Fitness characteristics of *P. lilacinus* strains under laboratory conditions

Strains	Growth rate (cm/d)	Conidiation (10^7 CFU/dish)	Egg parasitism (%)	Larval mortality (%)
P36-1	0.59^a±0.1	3.84^a±0.2	63.5^a±0.3	68.4^a±0.2
PL3	0.60^a±0.2	3.81^a±0.4	63.6^a±0.1	68.2^a±0.2

- The data are expressed as mean±SD

- Mean values with the same letter within single column are not significantly different by Duncan's multiple range test at 0.05 level

36-1. The mutant P100 achieved the highest persistence among the tested strains recording 5.9×10^6 spores/100 g soil in the third year. It also exhibited the highest virulence against root-knot nematodes recording 132 J_2/100 g soil and root gall index of 1.4 in the second and third year, respectively.

Discussion

To our knowledge, this is the first report demonstrating the full-length sequence and cDNA sequence of the β-tubulin gene in *P. lilacinus*. The β-tubulin genes of the *P. lilacinus* strains with different resistance to carbendazim were sequenced to confirm resistance mechanisms of the β-tubulin gene.

Extensive work has been conducted in the area of the β-tubulin gene resistance mechanism. The principle of benzimidazole resistance is closely associated with point mutations in the β-tubulin genes that change the structure of the fungicide-binding point (Gafur et al. 1998; Albertini et al. 1999; Peres et al. 2004; Chung et al. 2006; Davidson et al. 2006; Ziogas et al. 2009). T274I, R282Q, and Q292E mutations significantly weakened the interactions and binding of the drugs taxol and epothilone (Natarajan and Senapati 2012), while Q43P and R318W mutations had a significant impact on platelet physiology in immune thrombocytopenia (Freson et al. 2005; Kunishima et al. 2009; Navarro-Nunez et al. 2011). The genetics of carbendazim resistance in *Aspergillus nidulans* (van Tuyl 1977), *Ustilago maydis* (Ziogas and Girgis 1993) and other fungal species revealed that in most cases it was based on a single gene. The well-characterized amino acid substitutions E198A, E198V, E198K and F200Y were found to be responsible for this target-site insensitivity in carbendazim-resistant *B. cinerea* isolates (Yarden and Katan 1993; Leroux et al. 2002). The codon 198 of the β-tubulin gene in carbendazim-resistant *Neurospora crassa* is glutamic acid and codon 200 is phenylalanine (Fujimura et al. 1992). Mutation in position F167Y was observed in ruminant gastrointestinal nematodes such as *Haemonchus contortus* and *Trichostrongylus colubriformis* (Prichard 2001; Silvestre and Cabaret 2002), while some other resistant nematodes have a tyrosine residue instead of phenylalanine at position 200 of β-tubulin (Elard and Humbert 1999; Winterrowd et al. 2003). In the present study, the high-resistance mutants P50 and P100 also have a tyrosine residue instead of phenylalanine at position 200 of β-tubulin (Table 4). Moreover, the P100 mutant has two more points, S145A and T185A, of mutation leading to more

Table 6 Fitness characteristics of *P. lilacinus* strains under field condition

Treament	December 2012 to February 2013				November 2013 to January 2014				March 2014 to June 2014			
	Spores / 100 g soil	J_2/ 100 g soli	RGI	% yield increase	Spores / 100 g soil	J_2 / 100 g soli	RGI	% yield increase	Spores / 100 g soil	J_2/ 100 g soli	RGI	% yield increase
CK^-	–	664^c	5	–	–	678^c	5	–	–	588^c	5	–
CK	–	233^b	2.56	10.2^b	–	221^b	2.54	10.2^b	–	232^b	2.75	8.6^b
P36-1	0	235^b	2.55	10.2^b	0	217^b	2.51	10.5^b	0	212^b	2.5	9.5^b
P100	4.9×10^{6a}	156^a	1.66	20.5^a	5.3×10^{6a}	132^a	1.51	24.1^a	5.9×10^{6a}	139^a	1.4	24.5^a
PL3	5.1×10^{6a}	150^a	1.57	20.5^a	5.3×10^{6a}	135^a	1.55	22.3^a	5.4×10^{6a}	145^a	1.45	23.3^a

- Mean values with the same letter within single column are not significantly different by Duncan's multiple range test at 0.05 level

- J_2/100 g soil: second stage juveniles of nematodes/100 g of soil

- RGI (Root gall index): based on the mean percentage of the root system (20 plants) with galls where 0=no galling; 1=trace infection with a few small galls; 2=≤ 25 % roots galled; 3=26 to 50 %; 4=51 to 75 %; and 5 = >75 % roots galled

carbendazim-resistance level compared to P50. The amino acid substitutions are in well-correspondence with *Acremonium*, P41741.1; *Sclerotinia*, XP_001594844.1 *and Verticillium*, ABA61128.1 (Fig. 2). This gene alteration might be responsible for inhibiting the carbendazim binding ability to the target protein and consequently induce carbendazim-resistance.

The 3D homology model of β-tubulin developed in previous studies revealed that the mutation codons resulted in carbendazim-resistance of plant-pathogenic fungi include 6, 50, 134, 165, 167, 198, 200 and 257 (Robinson et al. 2004). Since S145A and T185A changes of *P. lilacinus* tubulin have never been demonstrated in literature as causing carbendazim resistance, their roles in this process were investigated. Our results confirmed that, neither S145A nor T185A (separately or jointly) induced resistance to carbendazim unless both were combined with F200Y substitution. Despite S145A and T185A substitutions did not induce resistance themselves; their conjugation with F200Y (P100) has doubled the carbendazim-resistance of *P. lilacinus* compared to the single substitution of F200Y (P50). The surface structure of P100 mutant may clarify this double-resistance since it was different from the wild strain *Pl*36-1 due to the amino acid substitution at these three sites. These surface structural changes might be a reason for producing more stable structure of β-tubulin gene leading to increased carbendazim resistance (Fig. 4).

In some fungi (e.g., *Botrytis cinerea*), most of carbendazim resistant strains with the change of E198A in the gene encoding β-tubulin are simultaneously very susceptible to diethofencarb such as phenotype Ben R_1 (Leroux et al. 1999). The discovery of this negative cross-resistance led to the application of a mixture of carbendazim and diethofencarb which gave rise to the change of F200Y simultaneously resistant to diethofencarb "phenotype Ben R_2" (Leroux et al. 1999; Petit et al 2010). While the carbendazim-resistant mutations of F200Y in *P. lilacinus* tubulin were first reported in this study, it was substantial to determine diethofencarb resistance according to this change, especially that this fungicide may be used in greenhouses on vegetable crops . Preliminary studies revealed that *P. lilacinus* 36-1, P50, P100, and PL3 were resistant (70 μg/ml) to diethofencarb. The hyphal growth and conidial germination of all tested strains were not affected by diethofencarb concentrations up to 1.0 μg/ml (Supplementary Fig. 2a and b). These findings were substantially in-line with Hwang et al (2009). Hwang's results showed a tyrosine instead of phenylalanine at the amino acid position 200 of the *C. gloeosporioides* tubulin producing resistance to carbendazim and "carbendazim+diethofencarb".

Regarding to resistance cost of β-tubulin transformant strains, deletion of *$\beta_2$tub* in *G. zeae* reduced conidiation, vegetative growth and pathogenicity decelerated growth and reduced hyphal branching (Qiu et al. 2012). Contrary to expectation that β-tubulin gene alteration may affects fungal fitness, our in vitro studies confirmed that the PL3 and SL3 mutants could grow normally in PDA containing 100 μg/mL carbendazim. Moreover, no significant differences were observed between the wild strain (*Pl*36-1) and the overexpressed strain (PL3) in linear mycelia growth, spore production and pathogenicity.

The present research gives an insight on improving the carbendazim-resistance of the nematophagous fungus, *P. lilacinus*. New amino acid substitution sites in β-tubulin gene have been analyzed for effective carbendazim-resistance. Amino acid substitutions caused by site-direct changes at particular target codons (145,185 and 200) were manifested to be the cause of fungicide resistance by loss or reduction of the binding affinity to benzimidazole associated with the amino acid changes in β-tubulin. Our findings, either in laboratory or field experiments, have led to three broad conclusions regarding β-tubulin gene alteration in the wild type, *P. lilacinus* 36-1. First, multi-mutation sites in the same gene can confer resistance. Second, the change F200Y combined with S145A and T185A induces a carbendazim-resistant mutant maintaining nematode pathogenicity. Third, the reduction of the nematodes measures combined with yield improvement introduces reliable evidence that P100 and PL3 can be considered as stable and successful strains in controlling root-knot nematodes in the field under carbendazim application programs.

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