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Generation of green fluorescent protein vector transformed Paecilomyces lilacinus Strains

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Paecilomyces lilacinus **is one of the most important biocontrol fungi and has been applied extensively for controlling root-knot nematodes. The protoplast/PEG/$CaCl_2$ method was used to generate green fluorescent protein (GFP) transformants of two strains of *Paecilomyces* lilacinus (strain 20-7 and 618). Two transformed strains were successfully generated and identified by fluorescence microscopy. Transformation was confirmed by recovery, cloning and sequencing of polymerase chain reaction (PCR) products and Southern blot analysis. Two stably transformed strains were isolated (20-7 and 618 strain). GFP transformants of *Paecilomyces lilacinus* provide useful resources for elucidating the mechanism of controlling root-knot nematodes.**

Key words: *Paecilomyces lilacinus,* green fluorescent protein, fluorescence expression, transformants.

INTRODUCTION

Paecilomyces lilacinus (Thom.) Samson 1974 is a common saprobic (typical soil-borne) filamentous fungus, and has been found in a wide range of habitats including soils and various kinds of plant roots, which is able to parasitize various plant parasitic nematodes. In the meantime, it is also a kind of natural enemy for(of) some insects. *P. lilacinus* was firstly discovered to infect the eggs of *Meloidogyne incognita* and *Globodera pallida* and females of *M. incognita* (Jatala et al., 1979), and has been isolated from eggs, egg masses, females and cysts of many plant parasitic nematodes. At present, its taxonomic status has aroused controversy and has been named newly as *Purpureocillium lilacinum* (Luangsa-ard et al., 2011). The study on biocontrol mechanism of *P. lilacinus* has kept on going deeper constantly. The process of infection by *P. lilacinus* involved interaction of physical and chemical ways (Holland et al., 1999; Liu et al., 2004), and it was reported that extracellular serine protease and chitinase played an important role in the process (Khan et al., 2003; Wang et al., 2010; Yang et al., 2011, meanwhile, *P. lilacinus* also has inhibitory effects on some plant pathogenic bacteria, Fungi and yeast (Guo et al., 2011). Besides, *P. lilacinus* fermentation liquid could promote the growth of plant to increasing production (Liu et al.,1991; Khan et al., 2002). *P. lilacinus* as a kind of biocontrol agents, features in efficiency, safety and long efficacy duration during the course of nematicidal process. Meanwhile, a lot of experiments showed that there was no significant difference in nematicidal activity between *P. lilacinus* and chemical nematocides. Sometimes, *P. lilacinus* displayed better efficacy compared to chemical nematocides (Vijaya et al., 2002; Gao et al., 1998). Currently, amount of nematologists have utilized *P. lilacinus* for prevention and control of various plant

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nematodes such as root knot nematodes and cyst nematodes, and promising effects have been observed. The nematocides derived from *P. lilacinus* own the characteristics of contact toxicity, epidemicity, environmental safety, thus show advantages in environmental compatibility and safety. More importantly, it was hard to be resistant for long-term treatment. Based on its excellent efficacy and safety, *P. lilacinus* is more advanced than traditional chemical pesticide. Actually, it has already been commercialized by Asian Technology Center of the Philippines and sold in the form of fungal agent in the name of "BIOCON". Taken together, *P. lilacinus* is regarded as one of important biocontrol agents with great prospects.

Green fluorescent protein (GFP) has been expressed in a wide variety of filamentous fungi including *Colletotrichum lindemuthianum* (Dumas et al., 1999), *Magnaporthe grisea* (Kershaw et al., 1998), *Aspergillus flavus* (Du et al., 1999), *Paecilomyces fumosoroseus* (Cantone et al., 1999), *Botrytis cinerea* (Li et al., 2006), *Phomopsis viticola (*Anco et al., 2009), *Trichophyton rubrum* (Dobrowolska et al., 2009), *Dactylellina cionopaga* (Yu et al., 2012), etc. As the reporter gene, GFP is able to develop imaging continuously in individual organisms and being inherited in fungi in a stable manner. Therefore, it is widely used in pathogenic mechanism studies of biocontrol fungi. Chen had transformed GFP into *Colletotrichum destructivum* and *C.orbiculare* to obtain transformants, the growth speed and phenotype of which were the same as that of the wide type (WT). In addition, the transformants did not show significantly changes in pathogenicity in the course of infection of tobacco compared to WT (Chen et al., 2003). Meanwhile, Skadsen had transformed GFP into a kind of pathogen named *Fusarium graminearum* to study the infection way of *Arabidopsis thaliana* (Skadsen et al., 2004). Thus, GFP has increasingly become an effective tool for in-depth knowledge and study of germination, growth and infection way of biocontrol agents under natural environment. Currently, GFP has been successfully applied in marking of some biocontrol agents such as *Trichoderma harzianum* (Zeilinger et al.,1999) *Pochonia chlamydosporia* (Atkins et al., 2004) and *paecilomyces fumosoroseus* (Cantone et al., 1999*)*, and has been used to study ecological issues of biocontrol agents. The development of effective biocontrol agents depends on a thorough understanding of the mechanisms of nematode infection by nematophagous fungi (Yu et al., 2012). For these reasons, with the improvement of research ideas, technology and research programmes for GFP, the application of GFP will be very meaningful for further in-depth study in this field.

The aim of the research was to introduce a GFP gene to two WT *P. lilacinus* strains using protoplast/PEG/$CaCl_2$ method and to obtain stable generic and highly expressed isolate that are morphologically stable and pathologically similar to the wild type, it could be used for not only pathogenic mechanism studies but also real-time monitoring population dynamics and evaluation of control effect studies.

MATERIALS AND METHODS

Fungal Strain

Single-spore isolates 618 and 20-7 (CFCC, Beijing, China) of *P. lilacinus* were maintained in 10% glycerol at -80°C until needed. Cultured on potato dextrose agar medium (PDA) for 10 days at 25°C.

Plasmid

Transformation plasmid pCam35S-GFP was obtained from Dr. Peng Youliang, China Agricultural University, Beijing, China. It contains GFP gene and G-418 resistance gene (hptII), both of them were driven by the K35s promoter.

Transformation

Preparation of protoplasts and transformation were performed through the protoplast/PEG/$CaCl_2$ method previously described by Cantone and Vandenberg (1999) with the following modifications. Conidial suspensions were prepared in steriled 0.1% Tween-80 at the final concentration of 1×10^7 conidia per milliliter. Conidia were cultured in liquid medium(3 g/L yeast extract, 240 g/L sucrose, 20 g/L dextrose, 200 g/L potato) for 6-9 h in a shaker-incubator with 200 rpm at 28°C until the germination rate reached to 80%. Spore walls were digested for 6 h with 5 mg/mL cellulase (Sigma, USA) and 5 mg/mL snailase (Sigma, USA). The pCam35S-GFP plasmid was digested with restriction enzyme *Not* I (Neb, USA). According to the method of Gillian et al. (2010)., five dilutions from the same sample of 10^2-10^5 were prepared. 100µL aliquots of each dilution (containing protoplasts) were plated and cultured on solid regeneration medium plates(3 g/L yeast extract, 240 g/L sucrose, 20 g/L dextrose, 200 g/L potato, 15 g/L agar) in the addition of G418 (250 mg/L) at 25°C for 3 days. Transformants were selected on solid regeneration medium plates. Single colonies cultured for two days were transferred onto fresh PDA plates containing G418 (250 mg/L^{-1}) and incubated at 28°C for 10 days for further selection.

Fluorescence microscopy

The mycelia were obtained by inserting alcohol-sterilized cover glasses nearby single colonies at an angle of 45-50°C, mycelia were collected from the cover glasses after cultured for 2-3 days. The colonies expressing *gfp* gene were daily observed with fluorescence microscope (Leica DFC 300FX) (excitation at 450-490 nm and emission at 520nm).

Stablity test and molecular analysis of transformants

Stablity test

Colonies with green fluorescence were considered as transformants, which were subcultured for 4 times on PDA containing G418 (250 mg/L) before being used for single conidial re-isolation. Conidia produced by the transformants were washed off from the culture plate and used for a conidial suspension of 1×10^3 conidia per mL, which (100 µL) was evenly distributed on each PDA plate containing 250 mg/L of G418 (Single colonies formed on the plates were used to verify the presence of green fluorescence and further

molecular analysis to confirm transgene stability prior functional analysis.

Molecular analysis

Putative transformants observed by fluorescence microscopy was confirmed by PCR identification (Cormack et al., 1997) and Southern blot analysis. Genomic DNA of *P. lilacinus* was isolated with the DNeasy Plant Mini Kit (Tiangen, Shanghai, China). PCR analysis of transformants was performed using GFP-F (5'- ATG GTG AGC AAG GGC GAG GA-3') and GFP-R (5'-TGT ACA GCT CGT CCA TGC CG-3') primers that amplified a 710 bp fragment of *gfp* gene during 30 cycles of 94°C for 30 s,58°C for 45 s and 72°C for 45 s. The PCR product was purified and cloned into Pmd18-T (TaKaRa, Japan) for sequencing (Invitrogen China, Shanghai). Southern blot analysis was performed on *Nco* I digested genomic DNA, resolved in 0.8% agarose gels and transferred to positively charged nylon membranes (Sigma, USA). Preparation of DIG-labeled probes, hybridization (50°C) and chemiluminescent detection were performed according to the manufacturer's instructions (Roche, Switzerland). An internal sequence of the *gfp* gene (710 bp) which was amplified using GFP-F (5'- ATG GTG AGC AAG GGC GAG GA-3') and GFP-R (5'-TGT ACA GCT CGT CCA TGC CG-3') was used as the hybridization probe.

Bioassay of transformants

Stable single-cell isolate transformants of *P. lilacinus* strain 20-7 and 618 with strong GFP expression and the wild-type isolates of the two strains were cultured on PDA, four plates per isolate, at 28°C for comparison in growth rate, sporulation quantity and Pathogenicity. The colony diameters were measured every other day until the plate was fully covered with mycelia (Table 1). Pathogenicity detection was performed according to Wang et al. (2012).

Transformation efficiency of transformants

Protoplasts suspension (100 µL) of *P. lilacinus* (20-7 and 618 at 10^4 conidia/ml were spread on PDA containing G418 (250 mg/L) at 25°C for 3 days. Each strain had 20 Petri dishes. The number of confirmed transformants was calculated based on the counts of fluorescent strains obtained by fluorescence microscopy which was confirmed by PCR amplification and southern blot hybridization results. The actual transformation efficiency of *P.lilacinus* was calculated as follows (Gillian et al., 2010):

Transformation efficiency = the number of confirmed transformants/the total number of conidias plated.

RESULTS

GFP expression in transformants

Ten transfomants of each *P.lilacinus* strains *(*20-7 and 618) from fifth generation were selected randomly and analyzed with fluorescence microscopy, respectively. As shown in Figure 1, GFP fluorescence was observed in all analyzed colonies. Transformants of 618 and 20-7 showed strong fluorescence. The level of GFP fluorescence in the transformants was stable even when the transformants were cultured without G-418 for many generations. In contrast, the WT control did not show GFP fluorescence.

Identification of transformants

Stability of transformants with G418 resistance

Several potential transformants and wild type (WT) of 20-7 and 618 were subcultured on PDA containing G418 (250 mg/L) for 72 h; all of the transformants expanded for about 2 cm on the screening medium, and WTs was inhibited by G418. Keeping G418 in screening PDA medium, all the tansformants can be subcultured on the medium for five generations, indicating that the potential transformants stably expressed GFP and owned stable genetic characteristics with G418 resistance.

Molecular detection

Ten transformants were subcultured on PDA and then selected randomly from each strain, and the presence of *gfp* gene was confirmed by Southern blot analysis using the *gfp* gene as a probe. To check the stability of the transformants, genomic DNA was isolated from mycelia cultured in non-selective medium. Figure 2 displays notable band of *gfp* gene in tranformatans but not in WTs by Southern blot, suggesting that the fifth generational transformants still retained the introduced DNA. Since only a single band of 710bp was detected after genomic DNA was digested with *NCo I* enzyme. It implied that *gfp* gene was integrated at unique position (Figure 2). Additionally, presence of *egfp* gene in the genome of these transformants were also confirrmed by PCR with GFP-F and GFP-R specific primers with a band of expected size of 710 bp yielded (Figure 3).

Bioassay of transformants

Growth and sporulation

Five stable single-cell derived transformants of each *P. lilacinus* strain with strongly GFP expression were selected randomly for morphological characterization. The transformants of *P. lilacinus* strains 20-7, 618, had similar growth rate to the wild-type isolates when cultured on PDA in absence of G418. Meanwhile, isolates and the single conidial cultures derived from them sporulated as well as the WT isolates. Under microscope, hyphae and conidia of the transformants could not be distinguished from that of the WT isolates (Figure 1).

Pathogenicity on M. incognita

On *M. incognita*, the transformants showed similar infection rate to the WT isolates. After interaction for 20 h, the spore concentration of the transformants and WT isolates reached to 10^7/mL, the infection rates for mono-spore cell stage, double cell stage, four cell stage, six cell stage, multi-cell stage, blastula stage, gastrula stage and tadpole

Table 1. The number of single colonies generated from GFP transformants selected at different dilution gradients.

Strain	Dilution gradient				
	Blank	10^2	10^3	10^4	10^5
618	No single strain	No single strain	No single strain	6-10 single strain	1-3 single strain
20-7	No single strain	No single strain	No single strain	6-10 single strain	1-3 single strain

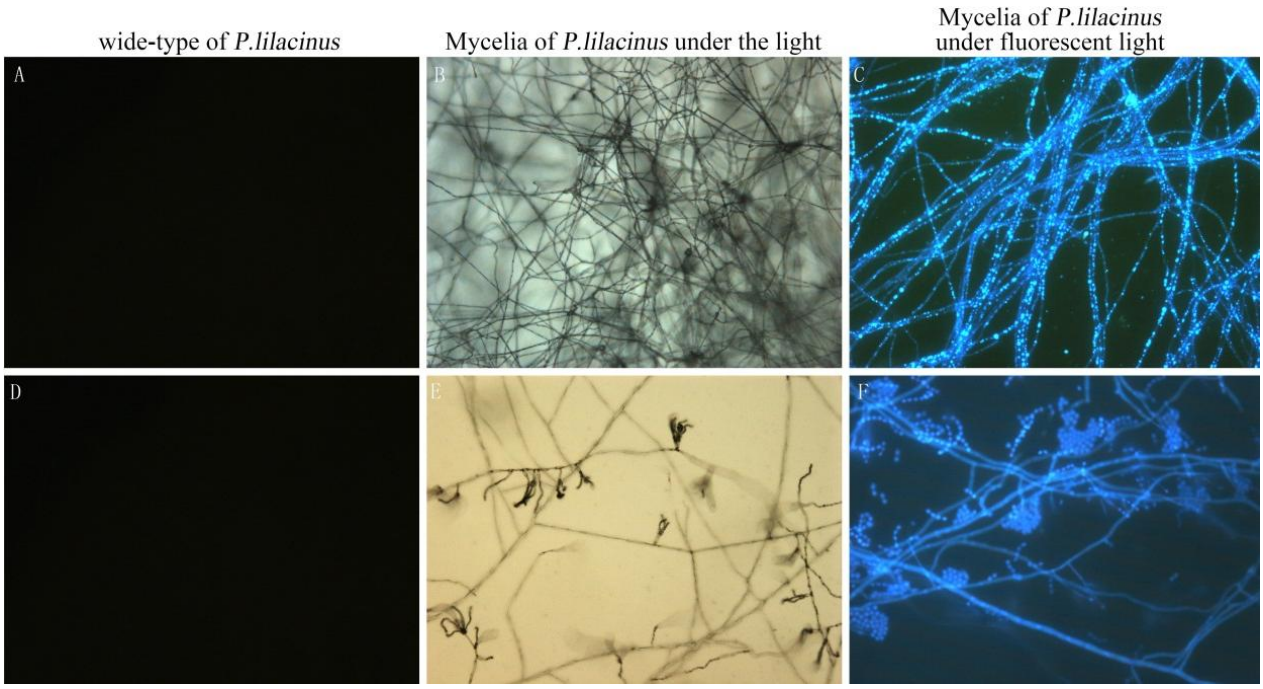

Figure 1. Expression of the green fluorescent protein in transformed 20-7 and 618 strains of *Paecilomyces lilacinus*. A,B,C: *P. lilacinus* 20-7; D,E,F: *P. lilacinus* 618.

Table 2. Transformation efficiencies of 20-7 and 618 transformants using G418 selection.

Plasmid	Total number of conidias	The number of confirmed transformant (number of dishes)/10μgDNA	Transformation efficient/10μgDNA
618	1×10^4	16	1.6×10^{-3}
20-7	1×10^4	19	1.9×10^{-3}

stage were all for 100%, while the fatality rate was 100%.

Transformation efficiency of transformants

The G418 resistant colonies appeared after culturing for 3-7days, then the conidia numbers of the two strains were calculated to compare efficacy of transformation. Both transformants of the two strains, 618 and 20-7, showed strong fluorescence, transformation efficiency of transformants were 1.6×10^{-3}/10μg DNA and 1.9×10^{-3}/10μg DNA, respectively. However, strain 20-7 was transformed with an higher efficiency than 618 under the same condition, but it did not make statistic differences (Table 2).

DISCUSSION

The efficient transformation system is an essential tool for gene manipulation and pathogenic mechanism study of *P. lilacinus*. PEG method is a kind of common method of cell transformation for filamentous fungi, and in the meanwhile *P. lilacinus* has phialospore conidia that are formed in the phialade and ejected from the cacumen (Shao et al., 1984). For this reason we decide to use *P. lilacinus* single spore conidia to prepare protoplasts in this study to improve the transformation efficiency.

Specific selection of the stable transformants is one of the most important steps of transformation methods.

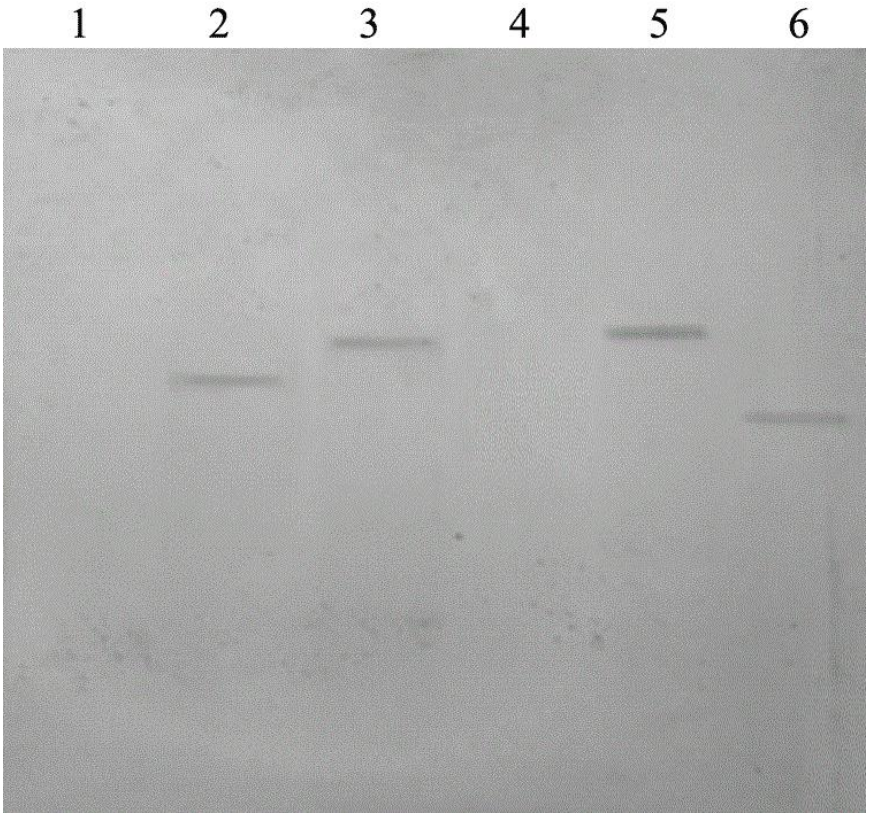

Figure 2. Southern blotting analysis of genomic DNA from *P.lilacinus* transformants. Lane 1: DNA sample from wild-type control of 20-7; lanes 2-3:DNA sample from 20-7 transformants; lane 4: DNA sample from wild-type control of 20-7; lanes 5-6: DNA sample from 618 transformants.

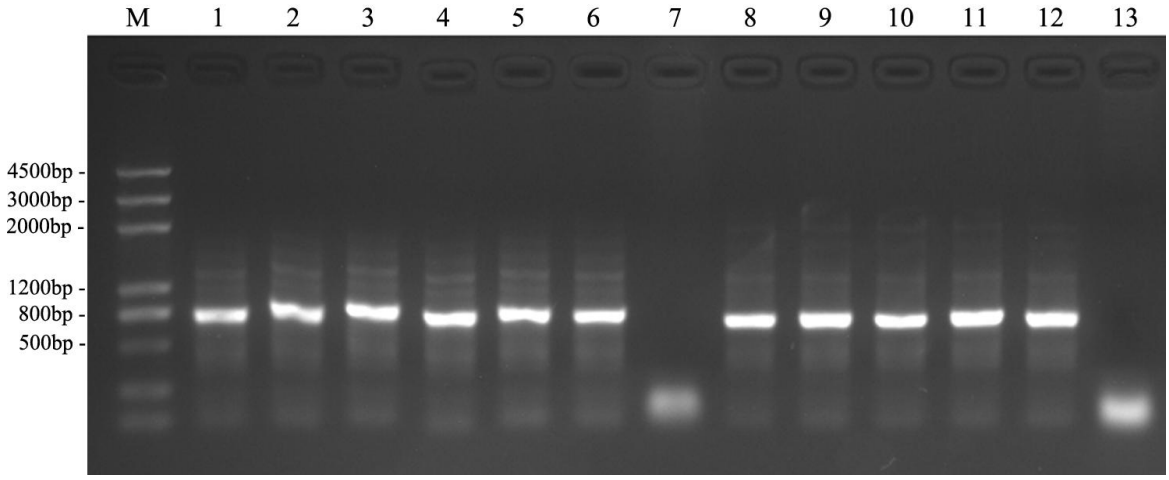

Figure 3. PCR analysis of genomic DNA from *P.lilacinus* transformants. Lane M: DNA marker; lane 1: DNA from pCam35S-GFP plasmid; lanes 2-6: DNA from 20-7 transformants; lane 7: DNA from wild-type control of 20-7; lane 8-12: DNA from 618 transformants; lane 13: DNA sample from wild-type control of 618.

Hygromycin B (HygB) has been widely used in transformations of filamentous fungi. However, the two *P. lilacinus* strains in the studies showed high resistance to HygB (data not shown). Consequently, HygB cannot be used as an antibiotics marker for selecting transformation of *P. lilacinus*. Based on our previous researches, we found

that *P. lilacinus* was very sensitive to G418, a number of neomycin family, then it was used screening of the transformation (Wang et al., 2010). Moreover, G418 is also consistent with established genetic transformation system of *Saccharomyces cerevisiae* by use of *aph* I and *npt* II (Guerra et al., 2006).

Strain 618 and 20-7 of *P. lilacinus* are two kinds of highly pathogenic strains obtained through selection (Wang et al., 2004). After interaction with *M. incognita* eggs for 20 h, when the spore concentration of the *P. lilacinus was* achieved to 10^7/mL, the infection rate for mono-spore cell stage, double cell stage, four cell stage, six cell stage, multi-cell stage, blastula stage, gastrula stage and tadpole stage is up to 100% and the fatality rate also reached to 100% (Wang et al., 2012). These rates were much higher than other reported *P. lilacinus* strains (Liu et al., 2006), thus these two strains have high value of application, and they can be used as alternative strains in production practice. In this study, we successfully established a genetic transformation system of *P. lilacinus* mediated with PEG methods using G418 as a selection marker. As expected, both 618 and 20-7 were successfully transformed with the *gfp* gene and the transformants showed strong fluorescence. For further investigation, the transformants exhibited stable expression of GFP after subculturing for several generations in PDA media containing G418. The results of PCR identification and Southern hybridization analysis also indicated that the fluorescence of these transformations was a result of *gfp* gene, which can be inherited and expressed in the transformation steadily.

The ultimate purpose of microbial gene marker is to obtain transformants with efficient and stably expressed marker gene in order to facilitate the analysis. Furthermore, it is suitable for observation and analysis of marked fungi of GFP by use of fluorescence microscope and FITC optical filter of confocal laser scanning microscopy exciting at 488 nm . All the transformants in this study demonstrated resistance to G418 and expressed high level of GFP during the whole course of development and reproduction. By use of these transformants, situations of *P. lilacinus* in plant roots such as colonization ability, occurrence and development etc can be studied real-timely. In addition, the impact of *P. lilacinus* on nematode can be further explored also, to provide an approach for studying evaluation of to evaluate control effect, study ecological tracking and assess environmental safety of *P. lilacinus* under different conditions.

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REFERENCES

Anco DJ, Kim S, Mitchell TK, Madden LV, Ellis MA (2009).Transformation of *Phomopsis viticola* with the green fluorescent protein. Mycologia.,101(6): 853-858.

Atkins SD, Mauchline TH, Kerry BR (2004). Development of a transformation system for the nematophagous fungus *Pochnia chiamydosporia*. Mycol. Res. 108(6):654-661.

Cantone FA, Vandenberg JD (1999). Genetic transformation and mutagenesis of the entomopathogenic fungus *Paecilomyces fumosoroseus*. J. Invertebr.Pathol. 74:193-197.

Chen N, Hsiang T, Goodwin PH (2003). Use of green fluorescent protein to quantify the growth of *Colletotrichum* during infection of tobacco. J. Microbiol. Meth. 53:113-122.

Cormack BP, Bertram G, Egerton M, Gow NA, Falkow S, Brown AJ (1997).Yeast-enhanced green fluorescent protein (yEGFP): A reporter of gene expression in *Candida albicans*. Microbiol. 143:303-311.

Dobrowolska A, Staczek P (2009). Development of transformation system for *Trichophyton rubrum* by electroporation of germinated conidia. Curr Genet. 55:537-542.

Dumas B, Centis S, Sarrazin N, Esquerré-Tugayé MT (1999). Use of Green Fluorescent Protein To Detect Expression of an Endopolygalacturonase Gene of *Colletotrichum lindemuthianum* during Bean Infection. J. Appl Environ Microbiol. 65(4):1769-1771.

Du W, Huang Z, Flaherty JE, Wells K, Payne GA (1999). Green Fluorescent Protein as a Reporter To Monitor Gene Expression and Food Colonization by *Aspergillus flavus* . J. Appl Environ Microbiol. 65(2):834-836.

Gillian B, Turgeon, Bradford Condon, Jinyuan Liu, et al (2010). Protoplast transformation of filamentous fungi. Moler and cell Biol. Methods for Fungi. 638:3-19.

Gao XB, Deng S, Zhou H, Zhang H, Chen C (1998). Efficacy of the MCWA18 strain of *Paecilomyces lilacinus* on *meloidogyne incognita*. Chin. J. Biol. Control. 14(4):163-166.

Guo ZB, Wang XZ, Wang LF, Zhu TH (2011). Preliminary Study on Antagonism of *Paecilomyces lilacinus* against Pathogen of Root Rot on Chinese Prickly Ash. Proceeding of the Annual Meeting of Chinese Society for Plant Pathology. Pp. 520-523.

Guerra OG, Rubio IG, da Silva Filho CG, et al (2006). A novel system of genetic transformation allows multiple integrations of a desired gene in *Saccharomyces cerevisiae* chromosomes. J. Microbiol Meth. 67(3):437- 445.

Holland RJ, Rita J, Williams, Keith L,Khan, Alamgir (1999).Infection of *Meloidogyne javanica by Paecilomyces lilacinus.* Nematol.1(2):131-139.

Jatala P, Kaltenbach R, Bocnagel M (1979). Biological control of Meloidogyne incognita and Globodera pallida on potatoes. J. Nematol.11:303-308.

Khan A, Williams K, Molloy MP, Nevalainen H (2003). Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. J. Protein Expr Purif. 32(2):210-20.

Khan M R, Kihara M, Omoloso AD (2002). Antimicrobial activity of *Michelia champaca*. Fitoterapia. 73(7-8):744-748.

Kershaw MJ, Wakley G, Talbot NJ (1998). Complementation of the Mpg1 mutant phenotype in *Magnaporthe grisea r*eveals functional relationships between fungal hydrophobins. J. EMBOJ. 17(14): 3838-3849.

Luangsa-Ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA (2011).*Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbio Lett., 321(2):141–149.

Liu XZ, Zhang KQ, Li TF (2004). Microbial control of plant-parasitic nematodes. Science and Technology Press, Beijing, China.

Liu XZ, Luo K, Zhang QW (1991). A preliminary bioassay of some physiological activities of fermentative filtrates of *Paecilomyces lilacinus*. Acta agriculturae universitatis pekinensis., 17(2):48-49.

Li XZ, Zhou T, Yu H (2006). Transformation of *Botrytis cinerea* with a green fluorescent protein (GFP) gene for the study of host-pathogen interactions. J. Plant Pathol. 6(2):134-140.

Skadsen RW, Hohn TM (2004). Use of *Fusarium geaminearum* transformant with gfp to follow infection pattens in barley and Arabidopsis. Physiological and Molecular. Plant Pathol. 64(1):45-53.

Shao LP, Shen RX, Zhang SX (1984). Fungal taxonomy. Forestry publishing house, Bejing, China.

Vijaya KN, Sujathamma P, Saviiyhti G, Murali MP (2002). Effect of biocontrol agents and Furadon 3G on the juvenile mortality of root knot nematode *Meloidogyne incognita*. Acta Agricalturae Zhejiangensis., 14(3):159-162.

Wang J, Liu F, Pan C (2010). Enhancing the virulence of *Paecilomyces lilacinus* against *Meloidogyne incognita* eggs by overexpression of a serine protease. J. Biotechnol Lett., 32(8):1159-66.

Wang XZ, Piao CG, Wang LF, Guo MW, Li Y (2010).Establishment of Agrobacterium tumefaciens-mediated transformation System of *Paecilomyces lilacinus*. Scientia Silvae Sinicae., 46(10): 95-102.

Wang XZ, Wang LF, Li Y, Guo MW (2012). Studies on Infection of *Paecilomyces lilacinus* on *Meloidogyne incognita.* Nematol. Res. China. 5(5):128-134.

Yang J, Zhao X, Liang L, Xia Z, Lei L, Niu X, Zou C, Zhang KQ (2011). Overexpression of a cuticle-degrading protease Ver112 increases the nematicidal activity of *Paecilomyces lilacinus*. J. Appl. Microbiol. Biotechnol. 89(6):1895-903.

Yu HY, Xue W, Gao XX (2012). Development of a transformation method for the nematophagous fungus *Dactylellina cionopaga*. Afr. J. Biotechnol. 11(14):3370—3378.

Zeilinger S, Galhaup C, Payer K, et al (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. J. Fungal Genet. Biol. 26(2):131-140.