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Agrobacterium tumefaciens-mediated transformation in the entomopathogenic fungus *Lecanicillium lecanii* and development of benzimidazole fungicide resistant strains

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ABSTRACT

Lecanicillium lecanii has been used in the biological control of several insects in agricultural practice. Since the gene manipulation tools for this entomopathogenic fungus have not been sufficiently developed, *Agrobacterium tumefaciens*-mediated transformation (ATMT) in *L. lecanii* was investigated in this study, using the wild-type isolate FZ9906 as a progenitor strain and the hygromycin B resistance (*hph*) gene as a selection marker. Furthermore, a field carbendazim-resistant (*mrt*) gene from *Botrytis cinerea* was expressed in *L. lecanii* FZ9906 via the ATMT system. The results revealed that the frequency of transformation surpassed 25 transformants/10^6 conidia, most of the putative transformants contained a single copy of T-DNA, and the T-DNA inserts were stably inherited after five generations. All putative transformants had indistinguishable biological characteristics relative to the wild-type strain, excepting two transformants with altered growth habits or virulence. Moreover, the resistance of the putative transformants to carbendazim (MBC) was improved, and the highest one was 380-fold higher than the wild-type strain. In conclusion, ATMT is an effective and suitable system for *L. lecanii* transformation, and will be a useful tool for the basic and application research of gene functions and gene modifications of this strain.

1. Introduction

The entomopathogenic fungus *Lecanicillium lecanii* is capable of infecting insect pests, has a broad geographical distribution, and shows promise in commercial development (Goettel et al., 2008). However, *L. lecanii* has a number of deficiencies with respect to field application, particularly low compatibility with fungicides (Zhai et al., 2013), which limit its use with fungicides in integrated pest management. Traditional breeding of entomopathogenic fungi includes physical or chemical mutagenesis, and protoplast fusion (Pu and Li, 1996), which are time consuming with high-resistant strains being difficult to obtain. Thus, genetic transformation could be a powerful means for the current problem solving.

To date, only one transformation method for *L. lecanii* has been reported. Hasan et al. (2011) described a conventional polyethylene glycol (PEG)-mediated transformation using the nitrate reductase gene as the selective trait. According to this study, low transformation frequency (about three transformants per μg plasmid DNA per 10^7 protoplasts) was a major drawback for the procedure employed. *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been employed for a wide range of plants (Zupan and Zambryski, 1995). It was reported that *A. tumefaciens* is also able to transfer its DNA to entomopathogenic fungi, including *Beauveria bassiana*, *Paecilomyces fumosoroseus*, and *Metarhizium anisopliae*, as well as to plants (dos Reis et al., 2004; Lima et al., 2006; Duarte et al., 2007). Compared with other transformation techniques, the ATMT method has been proved to increase transformation rate and show a greater degree of stability for the transgene (de Groot et al., 1998; Duarte et al., 2007), indicating that it can be an efficient tool for molecular manipulation of *L. lecanii*.

In this research, we evaluated the ATMT system with the hygromycin B resistance (*hph*) gene as a selection marker and determined the optimal conditions for *L. lecanii*. Furthermore, we successfully transferred a field MBC-resistant gene from *Botrytis cinerea* into *L. lecanii* and obtained some transformants with high-resistance to benzimidazole fungicides.

2. Materials and methods

2.1. Strains

L. lecanii strain FZ9906 was isolated by plate cultivation from soil collected at a tea garden in Fuzhou, China in 1999. FZ9906 conidia were collected by adding sterile water to the plates and rubbing the surface

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with the end of a sterile micropipette tip. Conidial suspension was filtered through four layers of Whatman lens cleaning tissue to eliminate mycelium. *B. cinerea* isolate CGY004 [field-resistant strain to MBC with a point mutation at position 198 (E198A) in the β-tubulin gene (GenBank accession no. U27198.1)] was kindly provided by Prof. Ming-Guo Zhou (College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China). *A. tumefaciens* AGL-1 was used for the transformation of *L. lecanii*.

2.2. DNA and RNA extraction

Fungal mycelia were ground to a fine powder in liquid nitrogen and incubated in DNA extraction buffer (100 mM Tris–HCl, pH 8.0, 20 mM EDTA, 2% CTAB, 1.4 M NaCl, 0.2% mercaptoethanol) at 65 °C for 30 min. The supernatants were treated with 10 μg of RNase (TaKaRa, Dalian, China), incubated at 37 °C for 1 h and subsequently extracted twice with an equal volume of phenol. The aqueous layers were extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1) and isopropyl alcohol was added to precipitate DNA. The precipitate was collected by centrifugation at 15,000 ×*g* for 15 min, washed with 70% cold ethanol and air dried. The pellet was resuspended in Tris–EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

RNA was extracted using TaKaRa RNAiso Reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Briefly, fungal mycelia were ground to a fine powder in liquid nitrogen and stood in RNAiso Plus for 5 min at room temperature. The supernatants were transferred into fresh tubes, added with 1/5 volume of chloroform, shaken vigorously and stood for 5 min at room temperature. After centrifuging at 15,000 ×*g* for 10 min at 4 °C, the upper aqueous phases were transferred into new tubes to which an equal volume of isopropanol was added, incubated for 10 min at room temperature, and centrifuged at 15,000 ×*g* for 10 min at 4 °C. After washing with 70% ethanol, the pellets were briefly air dried for 5 min and dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA was quantified by spectrophotometry at 260/280 nm. If necessary, RNA was treated with DNase I (TaKaRa, Dalian, China), extracted with phenol–chloroform, precipitated in isopropanol, re-suspended in DEPC-treated water and stored at −80 °C for the cDNA synthesis.

2.3. Plasmid construction

The pBHt2 plasmid (Mullins et al., 2001), carrying the *hph* gene under the *Aspergillus nidulans trpC* promoter, was used as the backbone of the binary vector. The *trpC* promoter (*PtrpC*) was amplified from the pBHt2 plasmid (Mullins et al., 2001) with the primer pair of TrpC-F/R. The coding sequence of the MBC-resistant β-tubulin gene (*mrt*) was cloned from *B. cinerea* isolate CGY004 with the primer pair of Mrt-F/R (Table 1). For the fusion of *PtrpC* and *mrt* fragment, an overlap PCR was performed as follows: 2 min at 98 °C, followed by 35 cycles of 10 s at 98 °C, 5 s at 58 °C and 1.5 min at 72 °C, and a final extension for 10 min at 72 °C. The *PtrpC*/*mrt* fusion fragment was released from pGM-*PtrpC*-*mrt* plasmid with Sac I and Hind III digestion and then ligated into pBHt2. The resulting plasmid was designated pBHt2-*PtrpC*-*mrt*. The protocols used for recombinant DNA work were described in Sambrook et al. (2001). The binary vector pBHt2-*PtrpC*-*mrt* was mobilized into *A. tumefaciens* AGL-1 by a heat-shock method (Bowyer, 2001).

Table 1
Oligonucleotide primers.[a]

Primer	Sequence (5′–3′)
TrpC-F	TGAGCTCTGATATTGAAGGAGCATTTTT (Sac I)
TrpC-R	TGGACAATCTCACGCATCTTGGGTAGAATAGGTAAGTCAG
Mrt-F	CTGACTTACCTATTCTACCCAAGATGCGTGAGATTGTCCA
Mrt-R	TATAAGCTTCTATTCCTCGCCCTCAA (Hind III)
TM-F	CTATTCTACCCAAGATGCG
TM-R	TAATGACCCTTAGCCCAG
PM-F	TCCTGGTACTGCTGGTATTCA
PM-R	AACTGGGCTAAGGGTCATT

[a] Sequences of the restriction site designed in primers are underlined and the corresponding restriction enzymes are indicated in parentheses.

2.4. Transformation

A. tumefaciens strain AGL-1, containing a binary vector, was grown at 28 °C for 18 h at 200 rpm in liquid LB medium supplemented with kanamycin (50 μg/ml). The *A. tumefaciens* cells were harvested, centrifuged and then diluted to (optical density) OD_{600} of 0.15 in induction medium (IM) (Bundock et al., 1995), with the presence of 0 to 800 μM acetosyringone (AS). The cells were grown for an additional induction period (0 to 10 h). The bacterial cell suspension (OD_{600} of 0.2 to 1.2) was mixed with an equal volume of a conidial suspension from strain FZ9906 (10^7 conidia/ml). This mix (200 μl per plate) was plated on a 0.45-μm pore, 45-mm diameter nitrocellulose filter (Whatman, Hillsboro, OR) and placed on cocultivation medium (CM) (same as IM except that it contains 5 mM glucose instead of 10 mM glucose) in the presence of 0 to 800 μM AS. Following incubation at 20 to 30 °C for 12 to 72 h, the filter was transferred to MM containing hygromycin B (250 μg/ml) as a selection agent for transformants and cefotaxime (200 μg/ml) to kill the *A. tumefaciens* cells. After 5 days of incubation at 25 °C, hyphae from visible fungal colonies were transferred to PDA agar plates containing hygromycin B (250 μg/ml) and incubated until conidiogenesis. To create monoconidial cultures, one germinating conidium from each transformant was picked and transferred to PDA containing hygromycin B (250 μg/ml) in a 5-ml plastic tube. Conidia from these monoconidial cultures were stored in 20% glycerol at −80 °C until further analysis. All experiments were carried out in triplicate, and the averages from three separate experiments are presented in the figures.

2.5. Identification of transformants

Transformants were grown for 3 days at 28 °C in 100 ml of liquid potato dextrose broth amended with hygromycin B (100 μg/ml) and cefotaxime (200 μg/ml). Mycelial mats were collected by vacuum-infiltration on a sterilized Whatman No. 1 filter paper and washed several times with sterile water before drying between paper towels. Total DNA and RNA of transformants were extracted as described above.

PCR analysis for detection of the *PtrpC*/*mrt* fusion fragment in putative transformants was performed using the primer pair of TM-F/R (Table 1). The PCR amplification protocol consisted of an initial denaturing cycle of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension for 10 min at 72 °C. When this was confirmed, reverse transcription (RT) was performed using M-MLV reverse transcriptase and oligo ($dT_{12–18}$) (Invitrogen, Carlsbad, CA) as previously described (Zhang et al., 2009), with the exception of fungal RNA. The mixtures were incubated at 37 °C for 50 min. Reactions were terminated by incubating at 70 °C for 15 min. PCR reactions were conducted in a volume of 25 μl containing 1-μl aliquots of RT products as template, 2.5 μl of 10× buffer, 1.5 μl of 25 mM $MgCl_2$, 2 μl of 1.25 mM dNTPs, 1 μl of primers PM-F/R mixture, and 1 U of *Taq* polymerase (Takara, Dalian, China). PCR was performed as follows: 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. All RT-PCR amplifications were performed in triplicate. To determine the T-DNA copy number, Southern gel blot analysis was performed with 15–20 μg genomic DNA digested with EcoRI for each sample. DNA probes were DIG-labeled according to the instructions of the manufacturer (Roche, Basel, Switzerland).