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Agrobacterium-mediated transformation as a useful tool for the molecular genetic study of the phytopathogen *Curvularia lunata*

Tong Liu · Lixing Liu · Xue Jiang · Jumei Hou · Kehe Fu · Feihong Zhou · Jie Chen

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Abstract In order to explore the molecular mechanisms of virulence and genetic variance of *Curvularia lunata* in maize, an ATMT (*Agrobacterium tumefaciens*-mediated transformation) system was established in order to create a wide range of insertional transformants of *C. lunata*. Our results showed that the germinating conidia were the ideal starting material for transformation. Based on our optimised transformation condition, the transformation efficiency of *C. lunata* with T-DNA was improved greatly, and the average transformation frequency was as high as 84±5 transformants per 1×10^6 germlings. Southern blotting results of 39 randomly-selected transformants showed a unique hybridisation pattern and predominant single-copy insertions. An ATMT library containing approximate 3000 transformants was generated, and four types of transformants were screened in terms of growth rate, sporulation, mycelial pigmentation, and toxin production *in vitro*. This library will be used to identify genes involved in the virulence of the pathogen.

T. Liu · L. Liu · X. Jiang · K. Fu · F. Zhou · J. Chen (✉)
Department of Resources and Environmental Sciences,
School of Agriculture and Biology,
Shanghai Jiaotong University,
800 Dongchuan Road,
Shanghai 200240, People's Republic of China
e-mail: jiechen59@sjtu.edu.cn

J. Hou
Department of Biology and Chemical Engineering,
Jindezhen Comprehensive College,
Jiangxi 333000, People's Republic of China

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Introduction

Curvularia lunata, a causal agent of leaf spot of maize, is distributed widely in the world and has been one of the major diseases in maize-growing regions in China in past decades (Dai et al. 1995, 1998; Li et al. 2006; Macri and Lenna 1974). In order to uncover the mechanisms for virulence and genetic variance of the pathogen, many studies have been focused on the identification of biochemical virulence factors such as toxin (Liu et al. 2009; Macri and Vianello 1976; Xiao et al. 2006) and cell wall-degrading enzymes (Feng et al. 2002). However, very little is currently known about the pathogenicity mechanisms of this fungus at the molecular level although some genes related to virulence have been cloned. For instance, *Brn 1* has been successful cloned from the pathogen and preliminary results have linked the gene to virulence in maize (Xu et al. 2007). To fully understand genetic variance and virulence factors, especially those responsible for toxin biosynthesis, development of an efficient mutagenesis system that can create a wide range of transformants is of great significance.

REMI (restriction enzyme-mediated integration) and ATMT (*Agrobacterium tumefaciens*-mediated transformation) have been applied to tag and identify genes in plants and fungi. In the past, REMI has been

extensively used to tag pathogenicity genes or to functionally study genes in numerous fungal pathogens such as *Fusarium oxysporum* (Inoue et al. 2001), *Gibberella fujikuroi* (Linnemannstons et al. 1999), *Alternaria alternata* (Akamatsu et al. 1997; Kodama et al. 1998), *Colletotrichum graminicola* (Thon et al. 2000), and *Magnaporthe grisea* (Balhadere et al. 1999; Liu et al. 1998; Sweigard et al. 1998). However, REMI may create a certain portion of untagged mutation that would result in deletions and chromosome translocations unlinked to the inserted plasmid in both the presence and absence of restriction enzymes (Balhadere et al. 1999; Epstein et al. 1998; Linnemannstons et al. 1999; Sanchez et al. 1998; Sweigard et al. 1998). Compared to REMI, ATMT has become a better choice for genetic modification of a wide range of fungal species (Michielse et al. 2005) due to its broad range of starting materials including protoplasts (Abuodeh et al. 2000; de Groot et al. 1998; Michielse et al. 2004), spores (Rogers et al. 2004) and mycelia (Combier et al. 2003), high efficiency of transformation and high percentage of single T-DNA insertion. Therefore, it is of great interest to construct an ATMT system specifically applicable to insertional mutagenesis of *C. lunata*.

Materials and methods

Strains, culture conditions and binary vector

A Highly virulent strain CX-3 of *C. lunata* was used as a recipient strain for fungal transformation. *Agrobacterium tumefaciens* strains AGL-1, EHA105 and LBA4404 (provided by Professor Dabing Zhang, College of Life Science Biotechnology, Shanghai Jiaotong University) were maintained on LB media. The pBHt1 plasmid was kindly provided by Professor Jingao Dong (Department of Plant Pathology, Agricultural University of Hebei, China) and was transformed into the bacterial stains by heat shock.

Preparation of geminating conidia and protoplasts of *C. lunata*

Germinating conidia were prepared by the following procedure: a monoconidial isolate of CX-3 was cultured on potato dextrose agar (PDA) for a week at 28°C in the dark, and the conidia were then harvested by washing the culture with sterile water. The conidial suspension was filtered through three layers of sterile cheesecloth to remove large mycelial debris and agar particles. The resulting conidial suspension was diluted to a final concentration of 10^6 spores ml^{-1} and spread onto cellophane placed on PDA plates. The plates were inverted and incubated for 3, 6 or 9 h at 28°C in the dark. The germinating conidia were collected by washing the cellophane paper with physiological salt solution. The final suspension for transformation was adjusted to 10^6 spores ml^{-1}.

Protoplasts were prepared as described (Michielse et al. 2004) with modification. One ml of conidial suspension collected from PDA was added to 100 ml potato dextrose broth (PDB) and incubated at 28°C for 18 h at 180 rpm. The mycelia was harvested by centrifugation for 10 min at 3000 *g*, and washed twice with physiological salt solution. One gram of mycelia was ground under sterile conditions, and resuspended in 25 ml of 0.7 M KCl phosphate buffer (pH 5.8) containing 2% lywallzyme (Guangzhou Microbial Culture Collection Centre, China) and 4% cellulose (Sigma, America). After 5 h of incubation at 30°C at 100 rpm, protoplasts were separated from cell debris by filtration through cheesecloth and collected by centrifugation for 10 min at 3000 *g*. Finally, the protoplasts were suspended in osmotic stabiliser (0.7 M NaCl, 10 mmol l^{-1}Tris-HCl (pH 7.5), 50 mmol l^{-1} $CaCl_2$) and used for transformation.

Transformation

Agrobacterium-mediated germling transformation was carried out as described (Covert et al. 2001) with modifications. AGL-1, EHA105 and LBA4404 carrying binary vector pBHt1 were grown on LB supplemented with kanamycin (100 μg mg^{-1}) for 18 h at 28°C at 200 rpm. The bacterial cells were diluted to an optical density at 600 nm of 0.2 in induction medium (IM). The cells were then grown in IM at 120 rpm for an additional 6 h at 28°C until it reached an optical density at 600 nm of 0.6, at which point it was mixed with an equal volume of the conidial suspension. Aliquots (250 μl) of the mixture were added onto cellophane placed on co-cultivation medium (IM containing 5 mM glucose instead of 10 mM glucose) for co-cultivation. The mixture containing germinating conidia and bacteria without plasmid served as the control. After co-cultivation, the

cellophane was transferred onto PDA containing hygromycin B (200 μg ml^{-1}), cefotaxime (200 μM) and vancomycin (120 μg ml^{-1}) (selection medium), and the plates were incubated for 6–7 days at 28°C.

The procedure for *Agrobacterium*-mediated protoplast transformation was as mentioned above with slight modifications. The mixture (250 μl) of protoplasts and bacteria with plasmids was plated directly on the co-cultivation medium. After co-cultivation, the selection medium was applied as a top agar and the plates were incubated at 28°C for an additional 7–8 days. All experiments were conducted in triplicate.

Analysis of mitotic stability of transformants

Individual hygromycin B-resistant colonies from the selection medium were transferred to PDA containing hygromycin B, and incubated until the growing edge of the hyphae reached the edge of the plates. Mycelial plugs were taken from the edge of the culture and transferred into 1 ml of sterile water in a 1.5 ml tube and vortexed vigorously to wash off the conidia. The resulting suspension was diluted 1000 times. An aliquot of 10 μl was streaked onto PDA containing hygromycin B, and incubated until colonies formed. Single-conidial colonies were isolated and maintained on the non-selective plate. To assess the mitotic stability of hygromycin B resistance, 200 randomly selected transformants were subcultured for five generations in the absence of hygromycin B, and then transferred to PDA containing hygromycin B.

Molecular analysis of transformants

To obtain genomic DNA of *C. lunata*, 39 randomly-selected transformants were grown in 100 ml potato-dextrose (PD) for 3 days at room temperature at 150 rpm. Mycelial mats were collected and washed several times with sterile water, then ground into a fine powder in liquid nitrogen. Genomic DNA was extracted as previously described (Rogers et al. 2004).

To confirm the insertion of T-DNA in the host chromosome, PCR amplifications were carried out by amplifying an internal 811 bp region of the *hph* gene located on the T-DNA using primers HPH-A: 5`-CGACAGCGTCTCCGACCTGA-3` and HPH-B: 5`-CGCCCAAGCTGCATCATCGAA-3`. The PCR reaction condition was designed as follows: 1 cycle at 94°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 2 min, and 1 final cycle at 72°C for 5 min.

Southern blotting analysis was performed to determine the frequency and randomness of T-DNA. Genomic DNA from the 39 randomly-selected transformants was digested with *HindIII*. The products were separated by electrophoresis on 1% agarose gel, and transferred to Hybond N + nylon membranes (Amersham, USA). The DNA fragments attached to the filter were hybridised to a labelled DNA probe (811 bp *hph* fragment) and detected using Gene Images Alkphos Direct Labelling and Detection System using manufacturer's instructions (Amersham, USA).

Screening of transformants

To identify genes involved in the virulence of *C. lunata*, transformants were screened using a toxin bioassay, based on scoring of lesion development. Conidial suspensions of each transformant grown on PDA were adjusted to 10^6 spores ml^{-1} with sterile water. The fourth leaves of susceptible maize seedlings (Huang zao 4) at the 7th leaf stage were cut into 2 cm slices and puncture wounds were made on the leaf surface along the vein; 5 μl of the conidial suspension from each transformant was then placed on the wounds, and the leaves placed on moistured filter paper in Petri plates. Conidial suspensions of CX-3 were used as the control. Symptoms on leaves were put into four grades: 0 = no symptoms; 1 = small necrotic spot with halo (diam ≤2 mm); 2 = necrotic spot with halo (diam 2 to 3 mm); 3 = necrotic spot with halo (diam 3 to 5 mm). The symptoms were recorded after 3 and 5 days of incubation at 25°C. The wild-type strain CX-3 typically caused grade 2 symptoms. Transformants showing less virulence than CX-3 were selected and cultured in Fries mediums to further examine the production of toxin as described by Liu et al. (2009). The culture filtrate (100 ml) was extracted with an equal volume of ethyl acetate. The extract was evaporated to remove the ethyl acetate, and then dissolved in ethanol (100 μl). Two microliters of the final extract from each transformant were spotted onto silica gel plates (Silica gel 60 F 254, Kongke, China) with solvent system butanol: acetone: water (4:5:1, v/v/v). The plates were visualised by UV irradiation (254 nm). Spots with R_f = 0.53 were considered as toxin produced by the transformants

(Liu et al. 2009). Extracts from wild-type strain CX-3 and non-inoculated medium were used as positive and negative controls, respectively.

Simultaneously, growth and morphology of transformants were compared with parent strain CX-3 through conventional methods. All hygromycin-resistant transformants were further divided into different groups based on growth rate, sporulation, and mycelial pigmentation.

Data analyses

All data were analysed using the SAS statistical package (SAS Institute 1989). Statistically significant analysis of variance (ANOVA) was further analysed using least significant difference (LSD) tests.

Results

Hygromycin B sensitivity of *C. lunata* and analysis of mitotic stability of the transformants

The sensitivity of wild-type CX-3 to hygromycin B was determined by transferring the pathogen onto PDA containing hygromycin B in gradational concentrations of 50, 100, 150, 200, 250 and 250 μg ml^{-1}. The growth of CX-3 was partially inhibited under the concentration of 100 μg ml^{-1} and was completely inhibited under 150 μg ml^{-1} for 14 days at 28°C. The resistant colonies grown on PDA containing 200 μg ml^{-1} of hygromycin were selected as transformants through an 8-day period. The growth and colony features of these transformants were not affected by this concentration of hygromycin compared to the transformant growth on PDA without it. Thus, 200 μg ml^{-1} hygromycin was chosen as the optimal concentration to screen transformants. The result showed that 198 out of 200 transformants randomly selected maintained their hygromycin resistance and 99% mitotic stability was confirmed by the consecutive subculture test. The transformants on the selection medium are shown in Fig. 1.

Optimisation of ATMT system for *C. lunata*

To establish a system of ATMT for *C. lunata*, ungerminated spores of the pathogen were initially employed as the starting material because they were easy to obtain. No transformants was obtained even though a number of parameters including cultivation time and temperature, co-cultivation conditions, *Agrobacterium* strains, concentrations of acetosyringone and different membranes for co-cultivation were used (data not shown).

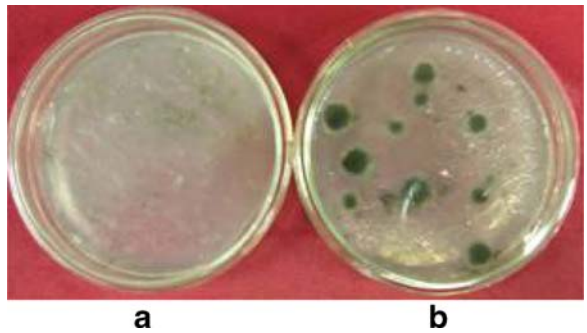

Fig. 1 Transformants of *C. lunata*. A: the mixture of the germlings and bacteria without plasmid. B: colonies of *C. lunata* from cellophane on selection medium containing hygromycin B (200 μg ml^{-1}), cefotaxime (200 μM) and vancomycin (120 μg ml^{-1})

When protoplasts were used as a starting material, a low number of hygromycin B-resistant clones (15±4 transformants with protoplasts 10^6 ml^{-1}) were obtained 24 h after co-cultivation. However, transformation frequency could not be determined due to their instability 2 days after co-cultivation. In comparison, much higher transformation efficiency was achieved when germinating conidia were used as a starting material. The transformation efficiency significantly depended on germination time. Significantly more transformants were obtained at 6 h. The efficiency was 91% ($P<0.05$) and 155% ($P<0.05$) higher compared to the efficiency at 3 and 9 h, respectively (Fig. 2). These results indicated that the conidia after 6 h of germination were the best starting material for transformation of *C. lunata*.

Different *Agrobacterium* strains also significantly impacted on the transformation frequency. AGL-1 yielded significantly more transformants than EHA105 and LBA4404. LBA4404 produced the least number of transformants ($P<0.01$) (Fig. 3).

Co-cultivation temperature and time were also accessed to optimise the transformation for *C. lunata*. Different combinations of co-cultivation temperatures (19°C, 22°C, 25°C, and 28°C) and times (24 h and 48 h) were used. At 19, 22 and 25°C, the number of

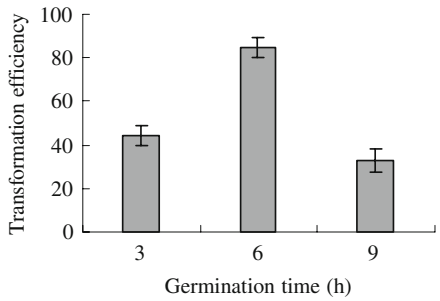

Fig. 2 The influence of different germination times of conidia of *C. lunata* on the efficiency of *A. tumefaciens*-mediated transformation. Conidia of CX-3 strain were co-cultivated with *A. tumefaciens* strain AGL-1 containing plasmids pBHt1 for 48 h at 25°C. Data presented are the averages of three experiments. Calculation of transformation efficiency for each experiment is based on the total number of transformants from eight plates

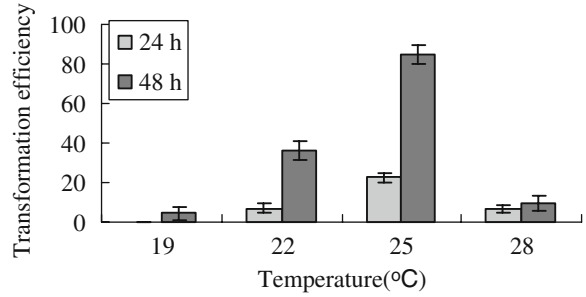

Fig. 4 Effect of co-cultivation times and temperatures on the efficiency of *A. tumefaciens*-mediated transformation. Conidia of CX-3 germinated for 6 h were co-cultivated with *A. tumefaciens* strain AGL-1 containing plasmids pBHt1. Data presented are the averages of three experiments. Calculation of transformation efficiency for each experiment is based on the total number of transformants from eight plates

transformants increased with co-cultivation time. At 28°C, transformation was very poor for both co-cultivation times. Co-cultivation time of 48 h at 25°C produced the highest number of transformants (Fig. 4).

Molecular analysis of transformants

The *hph* genes were detected with the specific primers from all 39 randomly-selected transformants by PCR, which confirmed the successful integration of T-DNA into the fungal genome (Fig. 5).

Southern hybridisation was performed to examine the integration mode of the T-DNA into the transformants. Of the 39 transformants, 33 showed only one hybridisation band (Fig. 6). The results suggested that ATMT, in most case, results in single-copy insertion into the host genome. The different band sizes showed that insertion occurred at different loci in the genome of *C. lunata.*

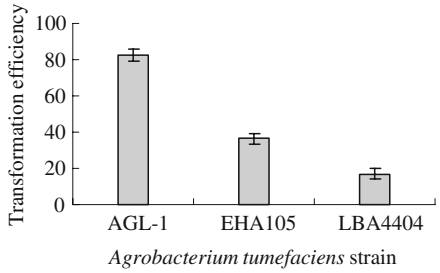

Fig. 3 Effect of different *Agrobacterium* strains on the efficiency of *A. tumefaciens*-mediated transformation. The conidia of CX-3 germinated for 6 h were co-cultivated with *A. tumefaciens* strains AGL-1, EHA105, and LBA4404, respectively, for 48 h at 25°C. Data presented are the averages of three experiments. Transformation efficiency of each experiment is the total number of transformants obtained from eight plates

Characteristics of transformants

With the optimised ATMT system, approximately 3000 transformants were obtained. They were divided into four groups based on sporulation, virulence, mycelial pigmentation and growth rate. From all transformants that showed normal colony features, five (T359, T801, T1879, and T2001) showed lower virulence than CX-3 based on the bioassay, and two (T359 and T801) produced no toxin. Transformants T2, T101, T408, T428, T806, T600, T950, T1101, T1845 and T2789 displayed a significant reduction in sporulation. Among them, T408 and T2789 also produced low amounts of toxic compounds and T806 produced no toxin. The growth rates of T 736, T1001 and T 1100 that appeared to have normal sporulation were significantly impaired ($P<0.01$). Varied colony features of these transformants are shown in Fig. 7.

Discussion

In this study, we describe the first application of *Agrobacterium*-mediated transformation to the important phytopathogen *C. lunata*. We also provide an efficient and stable transformation system using

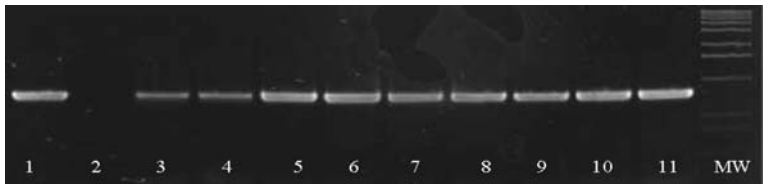

Fig. 5 PCR analysis of randomly-selected transformants of *C. lunata* CX-3 for the presence of *hph* gene. Lane 1: positive control with vector pBHt1; Lane 2: wild-type *C. lunata* DNA; Lanes 3–11: nine randomly-selected hygromycin B-resistant transformants; MW: 1 kb DNA molecular size marker

germinating conidia as starting materials. This approach could provide a tool of insertional mutagenesis for gene tagging in this fungus.

Our initial experiments found that conidia of *C. lunata* were difficult to transform by ATMT even though successful transformation using conidia as starting materials has been reported in other fungi. The thick conidial cell wall of the pathogen or cell melanins (Tsuji et al. 2003) might prevent T-DNA from entering the host genome. The similar phenomenon has also been observed in some other fungi including *Mucor circinelloides* (Nyilasi et al. 2005), *Rhizopus oryzae* (Michielse et al. 2004), *Phytophthora infestans* (Vijn and Govers 2003), and *Colletotrichum lagenarium* (Tsuji et al. 2003). Moreover, using protoplasts of the pathogen as a starting material is not applicable for ATMT transformation in this case. Our work demonstrates that germlings of *C. lunata* are the most suitable starting material for transformation as reported in *Ceratocystis resinifeera* (Loppnau et al. 2004), *Coniothyrium minitans* (Rogers et al. 2004) and *Coccidioides posadasii* (Kellner et al. 2005). It might be easier for T-DNA to enter the thin cell wall of germlings, which may lead to the high frequency of transformation. This assumption is supported by another observation during our study that using germlings removed from

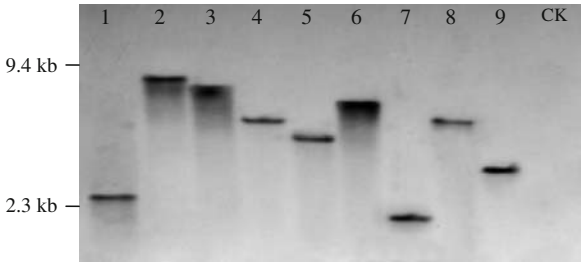

Fig. 6 Southern blot analysis of nine randomly-selected transformants of *C. lunata* and the original untransformed isolate CX-3. Lanes 1–9: randomly-selected transformants. Lane 10: untransformed wild-type *C. lunata*

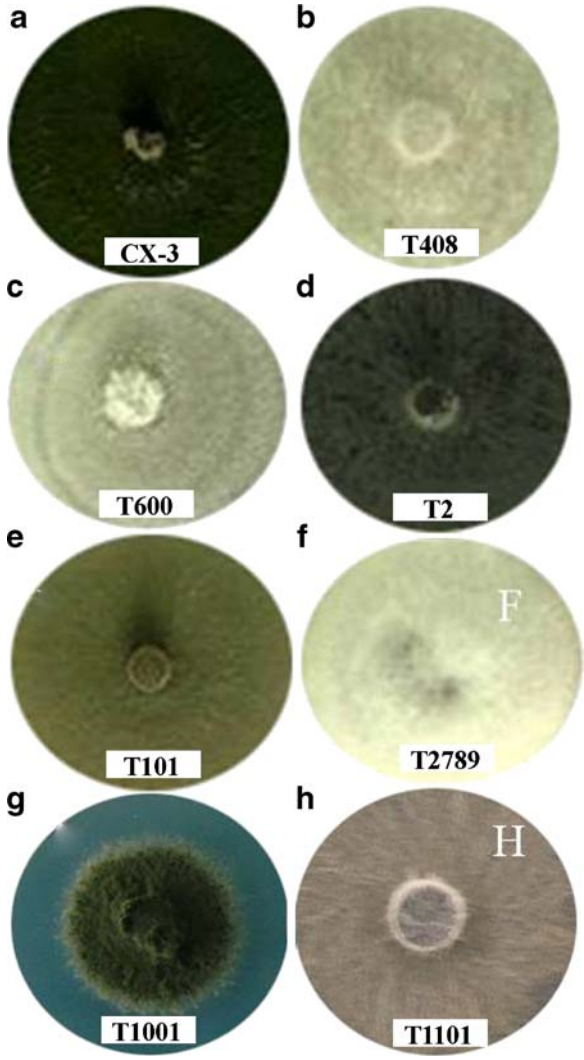

Fig. 7 Different colony features of *C. lunata* transformants selected from ATMT library. A: wild- type CX-3; B, C, F and H: transformants show reduced sporulation and varied pigmentation; D: transformant shows hyphae collapsed and significant reduction in sporulation; E: transformant shows reduced pigmentation and normal sporulation; G: transformant shows a reduced growth rate compared with wild-type. All transformants were cultured on PDA

cellophane would result in a higher transformation efficiency than using germlings in water. We speculate that minor injury caused by removal may help the T-DNA entry. Interestingly, the transformation efficiency was increased initially as was the extent of conidial germination, and then decreased at a later time. The phenomenon is also observed in the transformation with *Coniothyrium minnitans* (Li et al. 2005). The reason is still unknown.

Other factors should also be considered if a high-throughput transformation system of the pathogen has to be established. In this study, we have focused on different strains of *Agrobacterium* and co-cultivation conditions. *Agrobacterium tumefaciens* strain AGL-1 appeared to be more applicable for transformation of *C. lunata* with T-DNA than EHA105 and LBA4404. Co-cultivation time or temperature and combinations of these two factors can significantly influence the transformation process (Combier et al. 2003; Flowers and Vaillancourt 2005; McClelland et al. 2005). Several reasons might explain the importance of these two factors: (1) higher or lower temperature beyond the optimum range would result in the poor running of T-DNA transfer machinery of *A. tumefaciens*, (2) a prolonged co-cultivation time would make individual transformants difficult to isolate. Taken together, transformation frequency could be affected by multiple factors where biological status of the fungal pathogen appeared to be the most crucial.

For the construction of any large-scale insertional library, it is important that a high rate of single integration events occurs and the site of integration is random throughout the genome. Using germinating spores as staring materials have led to more multi-copy transformants in some fungi such as those reported by (Mikosch et al. 2001). However, a high proportion of single-copy transformants were obtained by the protocol described in this paper. Southern analysis of 39 randomly-selected transformants indicated that the rate of single-copy insertion was 84.6%. Additionally, the unique hybridisation pattern observed suggests that integration occurs randomly at different sites of the genome.

One of the main goals of creating an insertion library of *C. lunata* is to identify genes which may control toxin synthesis through toxin-deficient transformants. We conducted a simple *in vitro* bioassay to select the toxin-deficient mutants instead of isolating and purifying the toxin from each transformant in the entire mutant library. Although these data from the bioassay were reproducible, a high percentage of screened transformants did produce the toxin compound in the subsequent TLC analysis. The reduction of virulence in the transformants selected through the bioassay might be caused by changes in toxin quantity or other virulence factors. Only those transformants without the toxin spot on TLC analysis are considered as the toxin-deficient transformants in this study. In addition, several toxin-deficient transformants were also found with other changed features such as reduction in sporulation. We presume that some genes could not only regulate the toxin production, but also control the growth rate, sporulation and biology morphology. This hypothesis is supported by many studies (Cary and Calvo 2008; Hicks et al. 1997; Ochsner et al. 2009). Molecular analyses of the mutants described here and the specific genes involved in toxin biosynthesis are in progress.

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