Isolation and Identification of Pathogenicity Mutant of *Curvularia lunata* via Restriction Enzyme-Mediated Integration

Y. J. Wang · T. Liu · J. M. Hou · Y. H. Zuo

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Abstract In this report, 156 hygromycin-resistant mutants were generated via restriction enzyme-mediated insertional (REMI) mutagenesis. All mutants were subjected to a bioassay on detached leaves. Five mutants (T4, T39, T71, T91, and T135) showed reduced symptom development, whereas one mutant (T120) did not exhibit any symptoms on the leaves compared with the wild type. The pathogenicity of these mutants was further assayed through the spray inoculation of whole seedlings. The results demonstrated that the pathogenicity of the T4, T39, T71, T91, and T135 mutants was reduced, whereas the T120 mutant lost its pathogenicity. Southern blot analysis revealed that the plasmids were

inserted at different sites in the genome with different copy numbers. Flanking sequences approximately 550, 860, and 150 bp were obtained from T7, T91, and T120, respectively through plasmids rescue. Sequence analysis of the flanking sequences from T7 and T91 showed no homology to any known sequences in GenBank. The flanking sequence from the T120 mutant was highly homologous to MAPKK kinases, which regulates sexual/asexual development, melanization, pathogenicity from *Cochliobolus heterostrophus*. These results indicate that REMI and plasmids rescue have great potential for finding pathogenicity genes.

Keywords *Curvularia lunata* · REMI · Pathogenicity · Plasmids rescue

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Y. J. Wang
College of Life Science and Technology, Heilongjiang Bayi
Agricultural University, Daqing 163319,
Heilongjiang, People's Republic of China

T. Liu · J. M. Hou · Y. H. Zuo
Institute of Plant Pathology and Applied Microbiology,
Heilongjiang Bayi Agricultural University, Daqing 163319,
Heilongjiang, People's Republic of China

T. Liu (✉) · Y. H. Zuo
State Key Laboratory of Crop Stress Biology in Arid Regions,
Northwest A&F University, Yangling 712100, Shaanxi,
People's Republic of China
e-mail: liutongamy@sina.com

T. Liu · Y. H. Zuo
Key Laboratory of Plant Protection Resources and Pest
Management (Northwest A&F University),
Ministry of Education, Northwest A&F University,
Yangling 712100, Shaanxi, People's Republic of China

Introduction

Curvularia leaf spot in maize, caused by *Curvularia lunata* (Wakker) Boed, is one of the most serious diseases in maize production in China. It posed a great threat to maize production over the past decades. The disease occurred severely in Northeastern China in 1996, infecting nearly 40 % of the growing areas, leading to a grain loss of 260 million kg [1–3]. Although several recent studies have focused on breeding highly resistant varieties, virulence differentiation, and identification of virulence factors of this fungus such as cell wall-degrading enzymes, melanin, and toxins [4–7], relatively little is known about the molecular mechanism involved in the virulence and aggressiveness of *C. lunata* against maize.

Random mutagenesis studies have been effective in investigating complex biological processes, such as virulence and aggressiveness. A transformation technique called restriction enzyme-mediated integration (REMI)

allows the high frequency introduction of random tagged mutations into the host genome. This technique was originally developed for *Saccharomyces cerevisiae* [8] and later refined for *Dictyostelium discoideum* [9]. Due to the establishment of molecular techniques in a number of phytopathogenic fungi, REMI mutagenesis tagging has become an attractive method for the unbiased identification of pathogenicity genes. The major advantage of REMI is that it provides a method by which genes can be randomly disrupted via plasmid insertion, and it allows the subsequent cloning of these genes via plasmid rescue in *Escherichia coli* [10].

Numerous studies on transformation mutants such as ATMT and PEG-mediated mutants have been performed to obtain tagged mutations in *C. lunata* [11, 12]. However, little knowledge has been developed for the REMI transformation of *C. lunata* to produce pathogenicity mutants, particularly in terms of subsequent plasmid rescue for flanking sequences. In this study, genetic transformation was conducted using REMI and plasmids rescue, which has the greatest potential for finding novel gene-encoded determinants of pathogenicity in *C. lunata*.

Materials and Methods

Fungal Strains, Plasmid, and Culture Condition

A highly virulent isolate of *C. lunata* strain CX-3 from single spore germination, cultured on potato dextrose agar (PDA) medium at 28 °C, was used in this study as a recipient strain for fungal transformation. The plasmid pV2 (5,080 bp) containing the hygromycin and ampicillin resistance genes and was kindly provided by Prof. Jie Chen (Shanghai Jiaotong University), and was maintained on LB media containing 100 µg/mL ampicillin, and was used for transformation of *C. lunata*.

REMI Transformation and Acquisition of Transformants

Strain CX-3, grown on a PDA medium for three days at 28 °C, was used to prepare spore suspension (about 10⁶ spores/mL). The spore suspension was then transferred onto 50 mL of potato dextrose broth and incubated at 28 °C for 19 h at 150 rpm. The young mycelia were used to develop the protoplasts according to the methods described by Liu et al. [12]. The REMI transformation procedures were constructed according to [13]. Solid and liquid regeneration media were prepared according to previously described methods [14, 15]. The transformations were selected after 7 days of incubation on regeneration media containing 250 µg/mL hygromycin B for at 28 °C. The colonies were

subcultured five times on PDA supplemented with hygromycin B to obtain stable colonies, which were subsequently transferred to PDA medium. The chromosomal DNA from stable colonies were extracted and were used for the detection of *hph* (primers: HPH-A: 5'-CGACAGCGTCTCCGACCTGA-3' and HPH-B: 5'-CGCCCAAGCTGCATCATCGAA-3') and *amp* (5'-GGCGAAGAATCTCGTGCTTTCA-3' and 5'-CAGGACATTGTTGGAGCCGAAA-3') genes from plasmids. PCR amplification was carried out as described by [11, 16].

Pathogenicity Screening Tests

The pathogenicity of the primary transformants was assessed using a maize cut leaf assay. The cut leaf assay was performed as described by Liu et al. [11] with some modifications. Briefly, the fourth leaves of the susceptible maize seedlings (HUANG ZAO 4) in the 7-leaf stage were cut into 5 cm sections. Approximately 10 µL of conidial suspension (10⁶ spore/mL) was applied onto the surface of the leaves, which were then placed on moistened filter paper in Petri plates at 25 °C with 6-BA. The symptoms were recorded after 3 days of incubation at 25 °C using a standard method. Only the transformants with grades of 0 and 1 were selected for further assay via spray inoculation. The spray inoculation of whole seedlings was performed using the following methods. A conidial suspension (10⁶ spores/mL) containing 2 % sucrose and 0.02 % Tween-20 was prepared, and then sprayed onto maize seedlings using an air sprayer. The plants were inoculated for 24 h using a plastic bag with good moisture retention. All inoculated seedlings were grown for 7 days at 25 °C, a 14 h photoperiod, and 60 % relative humidity. The pathogenicity index was determined using the evaluation standard described by Chen et al. [17]. Wild-type *C. lunata* CX-3 was used as the control. The test was repeated three times using the same conidial concentration and the same inoculation conditions.

Molecular Analysis of the Mutants

Genomic DNA was extracted from the wild-type strain and pathogenicity mutants using the hexadecyltrimethyl-ammonium bromide (CTAB) methods described by Stewart and Via [18], and then digested with *Sac* I for 24 h at 37 °C. The *hph* DNA fragment amplified with primers HPH-A: 5'-CGACAGCGTCTCCGACCTGA-3' and HPH-B: 5'-CGCCCAAGCTGCATCATCGAA-3' was labeled with digoxigenin as probe, and then subjected to Southern blot analysis as previously described by Liu et al. [12]. Genomic DNA from the pathogenicity mutants with single-copy integration were digested with *Sac* I for 12 h in a 50 µL reaction mixture at 37 °C to recover pV2 and flanking genomic sequences from

the integration site. The same amount of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the enzyme digestion mixture, mixed thoroughly, and then centrifuged at $9,700\times g$ for 10 min. The supernatant liquids were subsequently transferred into a 1.5 mL tube and the same amount of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the procedure was repeated. The supernatant liquid was transferred into a clean tube, and 70 % ethanol was added. The precipitate was centrifuged at $5,900\times g$ for 5 min, and subsequently washed with 70 % ethanol. The precipitate was dissolved in 10 μ L of ligation buffer, and then ligated for 24 h at 4 °C using T4 DNA ligase. The ligation product was introduced into *E. coli* cells via electroporation. The plasmids were isolated from the resulting ampicillin-resistance transformants.

Results and Discussion

Acquisition of Transformants

Although transformation via REMI has been reported in several filamentous fungi, and the transformation conditions and their effects on plasmid integration have been studied, to our knowledge, the REMI protocol has not been used on *C. lunata* in previous studies. In this study, we successfully utilized the REMI protocol for the transformation of *C. lunata* with the transforming plasmid pV2. A total of 156 transformants were obtained using the REMI. To develop a highly efficient transformation system for *C. lunata*, we used different the concentrations of PEG, the

amount of protoplasts, and the restriction enzymes, but the transformation frequencies were not stable compared with those reported for other fungi. Therefore, REMI transformation is affected by more complication factors in this fungus, and still needs to be further studied.

To further confirm the transformants, seven randomly selected transformants were analyzed through PCR using the *hph* and *amp* gene primers. Two specific bands, 811 and 510 bp, were found in the seven transformants and the positive control pV2 via agarose gel electrophoresis (Electronic Supplementary Material 1). Therefore, plasmid pV2 was integrated into the chromosome of *C. lunata*.

Screening Pathogenicity Mutants

Curvularia lunata produces a number of pathogenicity factors during its interaction with plants. However, the mechanisms underlying its pathogenicity are still unclear. Therefore, the rapid isolation of pathogenicity deficient mutants for cloning the pathogenicity gene is very important. To screen the pathogenicity mutants by REMI, detached maize leaves from 7-leaf stage seedling were initially used to measure the ability of the mutant to infect host plants. The results revealed that five transformants (T4, T39, T71, T91, and T135) induced symptoms (areas surrounding lesion displays light maceration), and were classified as grade 1. One transformant (T120) did not induce any symptoms on detached leaves and was classified as grade 0; whereas wild type CX-3 caused symptom (area surrounding lesion shows significant maceration) with grade 3. The results are shown in Fig. 1. The

Fig. 1 Pathogenicity assays on detached leaves from wild-type strain CX-3, T4, T39, T71, T91, T120, T135 and one random selected transformant T140. *Ck* inoculation with distilled water

Fig. 2 Southern blot analysis of DNA isolation from wild-type strain CX-3, T4, T39, T71, T91, T120, T135 and one random selected transformant T140, digested with *Sac* I and probed with 811 bp *hph* gene fragment

Fig. 3 The amplification fragment from T4, T71, T91 and T120 by plasmids rescue. *M* 10 kb DNA marker

pathogenicity index was determined through standard evaluation. The average pathogenicity indices of T4, T39, T71, T91, and T135 were 24.5, 12.7, 15.4, 17.7, and 28.7 %, respectively, which demonstrated that T4, T39, T71, T91, and T135 are pathogenicity-reduced mutants. T120 showed no lesions on the leaves after 10 days of inoculation with the spore suspension, whereas the wild-type CX-3 reached a 33.1 % pathogenicity index. The results indicate that the T120 transformant lost the capacity to infect the host plant. Therefore, T120 transformant is a pathogenicity-deficient mutant.

Molecular Analysis of the Mutants

Plasmid rescue greatly facilitates the cloning of flanking sequences from mutants via REMI compared with other mutagenesis methods. This process has been successfully observed in some systems [19–23], but not in *C. lunata*. In

this study, plasmid rescue was also used to clone the flanking sequence from plasmid pV2. But, plasmid rescue has two main limitations that need to be solved. First is the insertion of plasmid in the host genome. Therefore, the screening pathogenicity mutants were analyzed through southern blot analysis to confirm the phenotype caused by the integration of the plasmids into the genome. The results showed that the T4, T71, T91, and T120 mutants only have one hybridization band, whereas the T39 and T135 mutants have two bands, respectively (Fig. 2). The results also demonstrate that the T4, T71, T91, and T120 mutant phenotypes resulted in single sites plasmid insertions into the genome of the transformants. To recover pV2 flanking sequences from the T4, T71, T91 and T120 mutants, their genomic DNA were digested with *Sac* I, which cleaves pV2 at a single site, and were then self-ligated with T4 DNA ligase. The ligation production was transformed into *E. coli*, and the positive clones were obtained on Luria–Bertani media containing ampicillin and sequenced. Approximately 550, 860, and 150 bp flanking sequences were obtained from T7, T91, and T120 were respectively obtained except for the vector pV2 sequence (Fig. 3) (Electronic Supplementary Material 2). No flanking sequence was obtained from T71. Sequence analysis of the T7 and T91 flanking sequences from showed no homology to any known sequences in GenBank. The flanking sequence from T120 was identified as highly homologous to *ChSte11* gene for Ste11-type MAPKK kinase, which regulates sexual/asexual development, melanization, pathogenicity from *Cochliobolus heterostrophus* [24]. These results show that REMI and plasmid rescue has the great potential for finding pathogenicity genes. The second limitation of plasmid rescue is the need to complement the mutant using cosmids that contain the corresponding region from the wild-type strain because of the large deletions in genomic DNA that is association with REMI transformation. In this study, whether the isolated sequence was responsible for the mutant phenotype still needs verification through gene disruption and complement.

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