Construction of a High-Quality Yeast Two-Hybrid Library and Its Application in Identification of Interacting Proteins with Brn1 in *Curvularia lunata*

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(Received on January 1, 2015; Revised on April 1, 2015; Accepted on April 7, 2015)

*Curvularia lunata* is an important maize foliar fungal pathogen that distributes widely in maize growing area in China, and several key pathogenic factors have been isolated. An yeast two-hybrid (Y2H) library is a very useful platform to further unravel novel pathogenic factors in *C. lunata*. To construct a high-quality full-length cDNA library from the *C. lunata* for application to pathogenesis-related protein-protein interaction screening, total RNA was extracted. The SMART (Switching Mechanism At 5' end of the RNA Transcript) technique was used for cDNA synthesis. Double-stranded cDNA was ligated into the pGADT7-Rec vector with Herring Testes Carrier DNA using homologous recombination method. The ligation mixture was transformed into competent yeast AH109 cells to construct the primary cDNA library. Eventually, a high qualitative library was successfully established according to an evaluation on quality. The transformation efficiency was about 6.39 × 10⁸ transformants/3 µg pGADT7-Rec. The titer of the primary cDNA library was 2.5 × 10⁷ cfu/mL. The numbers for the cDNA library was 2.46 × 10⁸. Randomly picked clones show that the recombination rate was 88.24%. Gel electrophoresis results indicated that the fragments ranged from 0.4 kb to 3.0 kb. Melanin synthesis protein Brn1 (1,3,8-hydroxynaphthalene reductase) was used as a “bait” to test the sufficiency of the Y2H library. As a result, a cDNA clone encoding VelB protein that was known to be involved in the regulation of diverse cellular processes, including control of secondary metabolism containing melanin and toxin production in many filamentous fungi was identified. Further study on the exact role of the *VelB* gene is underway.

**Keywords**: Brn1, *Curvularia lunata*, pathogenesis-related protein, VelB, Y2H library

The filamentous fungus *Curvularia lunata* (Wakker) Boedijn (teleomorph: *Cochliobolus lunatus* R. R. Nelson & F. A. Haasis) is a ubiquitous plant pathogen, which is causal agent of *Curvularia* leaf spot (CLS) (Macri & Lenna, 1974). Infection of maize leaves with *C. lunata* leads to huge yield loss in severe epidemic years in northern China. For instance, it occurred over 192,000 hm² and led to 8 million kg yield loss in Liaoning province in 1996 (Dai et al., 1995; Dai et al., 1998). Because of nationwide extensive application of resistance varieties containing tropic and sub-tropic germplasms, the incidence of disease infection and its severity were declined massively and less damage was observed in field. However, in recent years, the disease has bounced back again and caused serious damages in some maize growing areas such as Liaoning, Henan and Anhui province etc (Gao et al., 2014b). More importantly, methyl 5-(hydroxymethyl) furan-2-carboxylate (M5HF2C) produced by *C. lunata* in infected grains pose a serious threat to human and animal health (Gao et al., 2014a). Despite the high economic impact of CLS, efficient strategies for the management of CLS have not been fully developed, which could result partially from our limited knowledge on *C. lunata* pathogenomics.

In the last two decades, significant progress has been made towards a better understanding of the factors involved in the pathogenesis of *C. lunata*. A multiple virulence factors have been demonstrated to be involved in pathogen infection to maize, such as cellulose (Feng et al., 2002), non-host specific toxin (methyl 5-(hydroxymethyl)-furan-2-carboxylate) (Liu et al., 2009), melanin (Xu et al., 2007). It is worth mentioning that some of virulence related genes have been successfully cloned in previous work such as *Brn1* being required for DHN melanin synthesis (Liu et al., 2011), *Clt-1* regulating non-host specific toxin pro-
duction (Gao et al., 2015), two mitogen-activated protein kinases (MAPK) encoding genes (Clk1 and Cln1) (Gao et al., 2012; Wang and Chen, 2011). More recently, it has been reported that the Brn1 gene is not only responsible for melanin synthesis but also contributes, to some extent, to toxin production. Whereas, the related pathogenicity genes in melanin and toxin production have not been extensively studied.

A proteomic approach allows the simultaneous characterization of a large number of relevant proteins expressed by a genome (Conrad et al., 2008). Using the proteomic approach, researchers have successfully identified a Ctn1-regulated protein scytalone dehydratase (SCD, accession: KJ507954) associated with the production of toxin and pathogenicity in C. lunata (Gao et al., 2015). These studies are crucial in understanding the regulating network underlying pathogen virulence. However, the information presented in these studies can not directly reveal the proteins action mechanism of disease’s pathogenicity. Information on pathogenic factor interrelation and their interactions on each other is required for developing strategies to prevent fungal infection. Protein interaction networks are of fundamental importance to almost all biological processes, a powerful and high-throughput protein-protein Y2H screen system becomes more and more crucial to identify or mine the partners of proteins in the regulatory complexes (Miller and Stagljar, 2004).

To screen the pathogenesis-related interaction protein, an efficient and high-quality full-length cDNA library is necessary for studying the protein function and finding the protein associated with the pathopoiesia mechanism against host leaf. This study described a method for constructing a full-length cDNA library of C. lunata using the switching mechanism at the 5'-end of RNA transcript (SMART) technology. Moreover, melanin synthesis protein 1,3,8-hydroxynaphthalene reductase (Brn1 gene, GeneBank accession number: DQ358052) was used as a “bait” to test the efficiency of the Y2H library. This study not only demonstrated the critical techniques to construct a high-quality Y2H library but also provided informative cues to unravel interacting proteins with Brn1 involved in multiple secondary metabolic pathways in C. lunata.

Materials and Methods

Fungal strains used for the Y2H library. C. lunata CX-3 strain was selected for constructing cDNA library since it is highly virulent to maize and caused typical lesion on the maize leaves. The monoconidial culture of C. lunata was grown on potato dextrose agar (PDA) medium at 28°C (Gao et al., 2014a). The strain is stored in our laboratory and used for the study of pathogenicity mechanism of the pathogen for 10 years. The strain is maintained on PDA medium at 4°C or silicone beads at −20°C.

cDNA library primer. Universal primers were designed to amplify the inserted fragments of the cDNA library according to vector pGADT7-Rec sequence, and synthesized by Shanghai Sangon Company (Shanghai, China) as follows: MATCHMAKER 5' AD LD-Insert Screening Amplimer: 5'-CTATTTCGATGATGAAGATACCCCAACCC-3'; MATCHMAKER 3' AD LD-Insert Screening Amplimer: 5'-GTGAACTTGGGTTTTTCAGTATCTACGATT-3'.

Total RNA isolation. To extract fungal RNA, the strains were grown in 100 ml of PD medium in 250-ml Erlenmeyer flasks with continuous shaking at 160 rpm for 72 h at 28°C. The mycelia produced from these cultures were harvested by filtration using three layers of sterile cheese cloth, washed with sterilized double-distilled (dd)H2O, and ground in liquid nitrogen. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) from the powdered mycelia according to the manufacturer’s instructions, and then treated with DNaseI (Takara, Japan) to remove eliminate contaminated genomic DNA. The integrity of total RNA was analyzed by 1% agarose gel electrophoresis, followed by ethidium bromide (EtBr) staining. The concentration and purity of total RNA were determined by a spectrophotometer (Eppendorf AG, Hamburg, Germany) at 260 and 280 nm, respectively.

cDNA synthesis. The single-stranded cDNA (sscDNA) were synthesized using a SMART™ cDNA Library Construction Kit (Clontech USA, Mountain View, CA) according to the manufacturer’s instructions. sscDNA were synthesized by 1 μg of total RNA using SMART III oligo (5'-AAGCAGTGATCAACGACGATGTGGCATTATGGGCGG-3'), CDS III primer (5'-ATTCTAGAACCGAGCCGAGCTAGTGTGGCATTATGGGCGG-3'), and Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase. Double-stranded cDNA (dscDNA) were acquired by 20 cycles of long-distance PCR using Advantage 2 Polymerase Mix (Clontech USA, Mountain View, CA) with 2 μl of sscDNA product. The reaction parameters were as follows: pre-thermal denaturation at 95°C for 30 s; followed by 20 cycles of 95°C for 10 (extension/ enhance 5 s after each cycle), and 68°C for 6 min. To identify the quality of dscDNA, 5 μl of the PCR products was analyzed on 1% agarose gel electrophoresis, followed by