# Construction of a cDNA Library of *Pythium carolinianum*, a mosquito-killing fungus

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A mosquito-killing fungus was isolated in Guiyang City, China, in August 1994. It was preliminarily identified as *Pythium carolinianum* based on morphological studies. Observations on its extra cellular enzymes found that under the induction of chitin, it can produce high activity of substilisin-like proteases (Pr1) and trypsin-like proteases (Pr2). These are considered to be related with the process of by the fungus invading into mosquito body. In order to search for and make use of these genes, a cDNA library has been constructed. The methods used were mainly: 1) isolating total RNA of the fungus after 30 h induction of chitin; 2) using the total RNA as a template to synthesise cDNA using the SMART method; 3) inserting the double stranded cDNA into the vector, *Trip IEχ 2*, and 4) transforming *E. coli* strain *XL1—Blue* with the recombinant molecules to make an amplified library. The results of quality tests on the preliminary and amplified libraries showed that this cDNA library was successfully constructed. It can provide a direct source for screening genes of Pr1 and Pr2 and other proteases in the future.

**Key words:** mosquito biocontrol, Oomycetes, *Pythium*, Pr1, Pr2.

## Introduction

A new strain of fungus was obtained at Guiyang City in August 1994. It showed a high ability for killing mosquito larvae and a strong vigor against unfavorable conditions and has been preliminarily identified as *Pythium carolinianum* (Oomycetes, Peronosporales, *Pythiaceae*) mainly based on morphology of its hyphae and sporangia (Su *et al*., unpublished). In addition, when induced with chitin for about 30 h, the activity of Pr1 and Pr2-like enzymes, which are considered to regulate cuticle degrading proteases, increased markedly in the liquid culture. This indicates that this fungus has genes encoding these enzymes that are expressed under induction.

Fungi are presently not a well-exploited gene source, and therefore searching for fungal genes and making use of them to improve or even construct organisms that can serve as better biological control agents is highly significant. There have been studies on Pr1 and Pr2 enzymes in other organisms (e.g. St Leger *et al*., 1987). The overexpression of Pr1 gene was

used to improve the mycoinsecticide *Metarhizium anisopliae* (St Leger *et al.*, 1996). There is, however, little information available for these enzymes in *Pythium carolinianum*. In order to obtain genes encoding these enzymes, a cDNA library of this fungus after chitin induction has been constructed to be used as a gene source.

## Materials and methods

The isolate of *P. carolinianum* used was obtained from garden soil in Guiyang City and maintained on agar media of SFE (sunflower seed extract) and KPYG2 (mainly composed of peptone, yeast, glucose, cholesterol and some ions) with weekly subculturing, stored at 25 ± 1 C until needed.

### *Tests of Protease activities*

The chitin used for induction was made from lobster head. Ten grams (wet weight) of the fungal mycelia cultured in liquid SFE media for 3 d was filtered out, washed with sterilized water and transferred into flasks containing 100 ml of 1.5% chitin suspension, shaken (110 rpm) at 25 ± 1 C for 30 h. Its filtrate was diluted into different concentrations of 1:1, 1:10, 1:100, and 1:1000 with Tris-Hcl buffer (pH 8) to react with substrates, and $OD_{410}$ values were tested with spectrophotometer in 10 min.

### *Reagents*

- 1 Reagents for testing Pr1 and Pr2 activities:

Pr1: N-Suc-Ala-Ala-Pro-Phe-NA (Sigma Company)
Pr2: N-Ben-Phe-Val-Arg-NA (Sigma Company)

- 2 Reagents for construction of the library

MMLV reverse transcriptase (Amershampharmaica Biotech Company); SMART™ cDNA library construction kit; Advantage cDNA PCR kit; *λTripIEx2* (sfi A and B-digested arms); *E. coli XL1—Blue* (Clontech Company); λ-phage Packaging Reaction System (Promega Company); Oligonucleotide primers
(primer 1 5`-CTCGGGAAGCGCGCCATTGTGTTGGT-3`, primer 2 5`-ATACGACTCACTATAGGGGCGAATTGGCC-3`, Shanghai Sangon Company).

- Isolating the total RNA of *P. carolinianum* : Trizol-acid phenol method.
- Constructing the library: SMART™ cDNA library construction kit was used. The double stranded cDNA (ds cDNAs) were synthesized with cohesive ends with sfi sited (A and B), and were inserted into the sfi sites (A and B) of □ *TripIEx2* and then, transformed the *E. coli XL1-Blue* strain to construct the

preliminary library. An amplified library was built up for the stability of the information.

- Quality test of the libraries

The library titer, ligation efficiency and the length of the inserted cDNA fragments were tested.

**Results and discussion**

The protease activity of Pr1 and Pr2 like enzymes in the culture liquid of the fungus increased after being induced by chitin (Table 1). Pr1 and Pr2 activities are much higher in the liquid culture after chitin induction, than before induction. This suggests that the genes encoding these proteins are activated under chitin induction and should therefore contain a certain amount of corresponding mRNA in its total RNA. The cDNA library using this total RNA as a template should contain Pr1 and Pr2 genes and should be a good source of these and other related enzymes.

**Table 1.** A comparison of the absorbance at 410 nm of reaction substances of Pr1 and Pr2 before and after the induction of chitin.

| Enzyme and sample concentrations | Before induction | After induction |
|---|---|---|
| Pr1 | 1:10.2282 ± 0.0109 | 0.4620 ± 0.0021* |
| | 1:100.0220 ± 0.0017 | 0.0482 ± 0.0080* |
| | 1:1000.0028 ± 0.0012 | 0.0052 ± 0.0019** |
| | 1:10000.0015 ± 0.0008 | 0.0035 ± 0.0015** |
| Pr2 | 1:10.2287 ± 0.0100 | 0.4868 ± 0.0111* |
| | 1:100.0250 ± 0.0037 | 0.0450 ± 0.0033* |
| | 1:100 0.0088 ± 0.0016 | 0.0153 ± 0.0008* |
| | 1:1000 0.0048 ± 0.0017 | 0.0095 ± 0.0023* |

* $P<0.01$; ** $P<0.05$

The quality of the total RNA isolated from mycelia of *P. carolinianum* induced by chitin for about 30 h.

Uviol absorbance and electrophoresis methods were used to test the quality of the total RNA isolated from chitin induced fungal mycelia. The uviol absorbance test showed that the RNA purity and concentration were very high (Table 2). The results show A260/A280 values were between 1.8 ~ 2 fitting very well the normal test standard of total RNA (Lu, 1993). This indicates that the purity of total RNA is very good. In the electrophoresis gel in Fig. 1 there are two distinct bands, that is 18s rRNA and 28s rRNA. The rate of the brightness is about 1 ~ 1.5:1. The band of 5s rRNA is faint. This indicates that

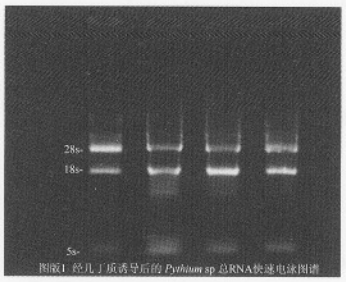


**Fig. 1.** The electrophoresis of total RNA (0.7 % Agarose, 15 v/cm).

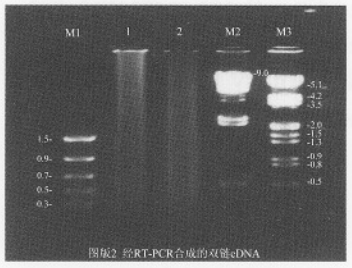


**Fig. 2.** Agarose gel electrophresis analysis of double strand cDNA synthesized with SMART™ method (1.1% agarose, 5 V/cm)
M1: PCR markers
M2: λDNA/HindIII markers
M3: λDNA/EcoR I+ HindIII markers
1: ds cDNA sample 1 (30 circles)
2: ds cDNA sample 2 (28 circles)

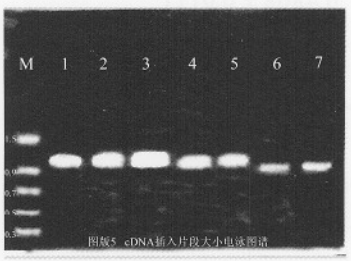


**Fig. 3.** Electrophoresis of the cDNA fragments ligated in *λTrip IEχ2* vector (1.1% agarose, 5 V/cm).
M: PCR markers
1~7:cDNA samples.

the completeness of the total RNA is good and the total RNA is satisfactory for cDNA library construction.

**Table 2.** The abforbances of *P. carolinianum* total RNA

| Sample | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| A260 | 0.075 | 0.080 | 0.051 | 0.143 |
| A280 | 0.040 | 0.042 | 0.026 | 0.068 |
| A260/A280 | 1.875 | 1.905 | 1.961 | 1.970 |
| Concentrations (ug/ul) | 0.75 | 0.80 | 0.51 | 1.34 |

The results of synthesized ds cDNA are showed in Fig. 2. The first strand of cDNA was synthesized with MMLV reverse transcriptase using the total RNA as template. The technique of switching mechanism at 5' end of RNA transcript enhanced the activity of reverse transcriptase. A modified oligo (dt) primer primes the first-strand synthesis reaction, and the SMART III oligo serves as a short, extended template at the 5' end of the mRNA. When the RT reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART III oligo which has a oligo (G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. The resulting full-length ss cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMART III oligo, which serves as a priming site (SMART anchor) in the subsequent amplification by RT-PCR (Clontech, 1998).

Only those ss cDNAs having a SMART anchor sequence at the 5' end can serve as a template and can be exponentially amplified. Incomplete cDNAs and cDNA transcribed from polyA(-)RNA will lack the SMART anchor and will not be amplified (Clontech, 1998). This selective amplification allows construction of a cDNA library using nanogram amounts of total RNA with a high percentage of full-length clones.

Two PT-PCR programs were used to obtain ds cDNA, that is 30 circles and 28 circles. The products were tested with electrophoresis and the results show "smear" at 0.1 ~ 8 kb site independently. This may be caused by non-homologous cDNAs and by-products (Belyavsky *et al.*, 1989). The brightness of the "smear" is the same as PCR marker or fainter, but it is fainter than M2and M3. Although standard ds cDNA fragments are at 0.5 ~ 10 kb sites from SMART kit, the fragments less than 0.4 kb are permitted to present. We can retain long cDNA strands through CHROMA SPIN-400 COLUMN.

The quality test results of the preliminary and amplified libraries are shown in Tables 3, 4 and Fig.3. The quality test for both preliminary and amplified cDNA libraries were made by counting the plaques and calculating

**Table 3.** The titer and recombination rate of the preliminary library.

| | 1 : 100 | 1 : 1000 | 1 : 10000 |
|---|---|---|---|
| Plaque number | 420 | 13 | 2 |
| Titer | $4.2 \times 10^7$ | $3 \times 10^7$ | $2 \times 10^7$ |
| Total titer | | $2.5 \times 10^7$ | |
| Blue plaque number | 2 | 2 | 0 |
| White plaque number | 32 | 15 | 8 |
| Ligation efficiency (%) | 93.75 | 86.87 | 100 |

the titer of the phage (pfu/ml). Usually, a library has titer value bigger than $1 \sim 2 \times 10^6$ pfu/ml containing enough mRNA information (Maniatis *et al.*, 1989). In this study, the titer of preliminary library is $2.5 \times 10^7$ pfu/ml. The results of recombinant phage screening (blue/white screening) show ligation efficiency was more than 80%. Usually, when the inserted cDNA fragments are short, a cDNA library cannot include complete coding information. We choose seven white clones to amplify with PCR. Electrophoresis results of the products show the lengths of the inserted cDNA fragments were more than 1 kb and there were length differences among the fragments. In order to stablelise the information of the library, we amplified the library. Its titer was $1.1 \times 10^9$ pfu/ml (usually $\sim 10^{10}$ pfu/ml is good (Maniatis *et al.*, 1989). According to these results, we consider that the quality of the library is good.

**Table 4.** The titer of amplified library.

| Samples | 1 | 2 | 3 |
|---|---|---|---|
| Plaque number | 960 | 900 | 600 |
| Titer | $\sim 2 \times 10^9$ | $\sim 1 \times 10^9$ | $3 \times 10^8$ |
| Total titer | | $1.1 \times 10^9$ | |

Since the cDNA library is constructed with the mycelia of *P. carolinianum* after being induced by chitin, it might contain more genes relating with mosquito-killing expression. We can screen genes encoding Pr1 and Pr2 from this cDNA library by using known gene fragments as probes, and, also, we can find more about other.

**Acknowledgements**

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