Construction and characterization of a genomic library of *Colletotrichum horii* from persimmon

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A cosmid library from genomic DNA of *Colletotrichum horii*, the fungal pathogen causing persimmon anthracnose is constructed. We established a extraction method for obtaining 145-250 kb genomic DNA, which is suitable for a cosmid library construction. The cloning procedures were as follows: mycelia was obtained following conidial germination, smaller fragments were removed from purified DNA through capillary ligation of the DNA to the cosmid vector, and *in vitro* packaging into the lambda phage. The library presently consists of 4.78×10^4 independent clones with an average insert size of about 34 kb. This cosmid library is the first to be constructed for cloning the pathogenesis related genes of *C. horii* and for analyzing the molecular and genetic characteristics of this species.

Keywords – anthracnose – DNA purification – genomic DNA – pathogenesis

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Introduction

Anthracnose of persimmon is a destructive disease (Zhang 2008, Xie et al. 2010) The disease is severe in persimmon nurseries and in orchards, causing serious economic losses to growers and has become a central problem in the development of persimmon industry in China (Zhang 2008, Xie et al. 2010). The pathogen causing this disease was identified previously as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Zhang et al. 2005b) and subsequently named *C. horii* (Hyde et al. 2009, Weir & Johnston)**.** Intra-cellular infection structures revealed during the infection processes have shown that *C. horii* is a hemibiotrophic species on Persimmon. During the biotrophic stage in persimmon , *C. horii* infection vesicles and primary hyphae are surrounded by an interfacial matrix that separates the fungal cell wall from the invaginated host plasma membrane, closely resembling that of *C. lindemuthianum* on bean (Zhang et al 2003, Zhang et al 2005a, Zhang 2008, Xie et al. 2010). *C. horii* provides an excellent pathosystem for studying the molecular basis for infection structure differentiation and fungal-plant interactions. However, the mole-cular mechanisms of interactions between the pathogen and host have not been investigated.

To understand fungus-host interactions, mutants related to pathogenesis-related genes

were obtained by *Agrobacterium tumefaciens*-mediated transformation and their fragments relating to pathogenesis-related genes were cloned (Sun et al 2008). For genes isolation and structure analysis it is important to construct a cosmid library. Here we report on a genomic library constructed for *C. horii* isolate TSG001 using SuperCos1 Cosmid Vector and its molecular characterization.

Material and Methods

Fungal material and culture

Colletotrichum horii isolate TSG001 in this study was isolated previously from *Diospyros kaki* cv. Wuheshi in Chunan area, Zhejiang province, China (Zhang et al 2005b). It was routinely stored with mycelial plugs (5mm) [glycerol-water, 20% (vol/vol)] at −70°C.

Plugs were removed from cryotubes and transferred to Petri dishes containing potato dextrose agar (PDA). Colonies were grown at 25°C under a near UV light with a 12 h photoperiod. Conidia were collected from cultures from 8 day-old cultures and washed twice with sterile water, and resuspended to 1×10^6 conidia/mL in sterile water. A 50 μL aliquot of conidial suspension was dropped into a flask containing 300 mL PDB (potato dextrose broth) and fungal mycelia were produced by shaking (180 r/min for 48 h at 25℃). Mycelia were collected on sterile filter papers for subsequent DNA extraction.

Preparation of high-molecular-weight DNA

5g mycelia were submerged in liquid nitrogen and ground into a fine powder in by using a mortar. The powder was transferred into a 50 mL centrifuge tube and 20 mL of 1 × TEN9 buffer(50 mmol/L Tris.CL (pH 9.0), 100 mmol/L EDTA-Na_2，200 mmol/L NaCL) was added. After the powder was dissolved thoroughly, 200 μL RNase stock (0.1 μg/μL) were added, and then 1 mL of 20% SDS and 1 mL proteinase K (10 mg/mL) were added into the tube, which was incubated on a rocker platform at 37°C for 24 h. To remove the proteins from the sample, phenol extraction was performed, using equal Vol. phenol: chloroform: isoamylalcohol (25:24:1). After the sample was centrifuged, supernatant was transferred into a new tube and phenol extraction was repeated two times. The aqueous phase was removed into dialysis bag, which was placed into TE (10 mM Tris-HCL pH 8, 1 mM EDTA) (1:1000 v/v) for 2 h at room temperature and then at 4°C overnight. The sample was transferred into a new tube from dialysis bag, and 1/10 vol. of 3M NaAc (pH 5.2) and 1 vol. isopropanol were added. The precipitated DNA was picked out by capillary, washed twice with 70% ethanol and was resuspended in 2 mL TE. The quality of the genomic DNA was checked by pulse field gel electrophoresis (PFGE) in a CHEF Mapper XA system (Bio-Rad Laboratories) at 14°C for 18 h. Lambda ladder PFG marker (New England Biolabs, USA) was used as a molecular size marker.

Insert DNA preparation

Partial digestions of the genomic DNA were carried out with different amounts of enzyme *Sau* 3AI (Takara, Japan) and digestion time. The optimal conditions firstly were established for obtaining The DNA fragments of 30-42 kb in length, and then a large scale digestion was performed for preparing insert DNA. After digesting, the DNA fragments was extracted with phenol–chloroform, precipitated with sodium acetate and ethanol, and then dephosphorated by 5 μL (0.5 U/μL) CIAP (Calf intestinal alkaline phosphatase, Promega, USA) at 37°C for 1 h. Subsequently, the reaction in samples were terminated by adding 1 μL of 0.5 M EDTA and incubated at 68 °C for 10 min. The samples were followed by phenol/chloroform and chloroform extraction, ethanol precipitation, and resuspension in water.

At the same time, the Super Cos1 vector used in this study was linearized by *Xba*I (Takara, Japan), dephosphorylated By CIAP (Promega), digested by *Bam*HI (Takara, Japan) and then two DNA fragments was purified by Axyprep PCR Cleanup kit (Axygen, USA), respectively, according to the manufacturer's instructions.

In vitro packaging

The DNA fragments of 30-42 kb were ligated into the Super Cos1 vector by T_4-DNA Ligase (Takara, Japan). The ligations were packaged *in vitro* into the λcl185 Sam7 Lambda (supplied in the Gigapack XL-11

Packaging Extract Kit) and plated with the *Escherichia coli* strains XL1-Blue MR onto NZY medium according to the manufacturer's instructions.

Quality of the packaged cosmids

The titer of the packaged cosmids was analyzed, adding 10 μL of the packaged cosmids with a serial dilution to 100 μL of he bacterial plating strain XL1-Blue MR host cells (supplied in the kit), and then the packaged cosmids were absorbed at room temperature for 30 min. The infected bacteria were spread on LB-ampicillin selection plates containing 50 μg/mL ampicillin and incubated at 37°C for 16 h. The colonies were counted and the titer (colony forming units, cfu/mL) was calculated according to the equation as follows: Titer (cfu/mL) = [(number of colonies) × (dilution factor) × (1000 μL/mL)]/(volume of phage plated in μL).

To assess the insert size and transformation efficiency, an aliquot of the infected bacteria was plated onto an LB-ampicillin plate. After incubation at 37°C for 16 h, individual colonies were inoculated into 5 mL of LB broth containing 250 μg of ampicillin and cultured at 37°C for 16 h with shaking. The cosmids was extracted from 1.0 mL of the culture using the CTAB method (Sun and Zhang, 2009), resolved in 0.1 mL TE (1 × TE contains 10 mmol/L Tris- HCl (pH 8.0) and 1 mmol/L EDTA-Na2), and analyzed with a 0.7 % agarose gel.

For average insert size investigation, 50 clones were randomly chosen; cosmid DNA prepared from each clone was digested with *Not*I (Takara, Japan) and *Eco*RI (Takara, Japan) and DNA fragments were checked by gel electrophoresis. Bands were visualized under UV light after EB staining.

Amplification and storage

Amplification of the packaged cosmids and storage in sterile glycerol were performed as recommended by the manufacturer.

Library characterization

To test stability of the library, one aliquot amplified cosmid library was taken and serial dilutions were performed. 30 colonies were randomly picked out and transferred into 5 ml of LB medium separately and cultured for 14 h. 0.8 ml from each culture was dropped into a new tube and added sterile glycerol to a final concentration of 15% and stored at -70 ℃ for 1 d. The tubes were thawed and then 50 μL was taken out from each culture, inoculated into another fresh 5 ml of LB medium and cultured for 16 h. Each clone was cultured in this way successively for 3 times. The cosmids was extracted from 1.0 mL of the culture using the CTAB method as described above and analyzed with a 0.7 % agarose gel.

Results

Preparation of genomic DNA and partial digestion conditions

A relatively high amount of high molecular weight genomic DNA was isolated from mycelia produced from conidia of *C. horii* and 1.0 mg genomic DNA was obtained. Analysis of pulse field gel electrophoresis showed that the majority of DNA fragments ranging between 145 and 250 kb were yielded (Fig 1), which was suitable for the requirement for construction of the library (about five times insert DNA size). The partial digestion conditions with *Sau* 3AI that yielded the majority of DNA fragments in the 30-42 kb range were established when 10 μg genomic DNA was digested with 0.5 μl *Sau* 3A I (0.2 U/μl *Sau* 3A I) for 5 min and this conditions were used to digest a large amount of genomic DNA (100 μg).

Quality of the packaged cosmids

After the digested DNA fragments were ligated, packaged plated with the *Escherichia coli* strains XL1-Blue MR, the colonies were counted and the titer was calculated. The result for titering cosmid library showed that the titer of packaged cosmids was 9.6×10^3 cfu/mL (Fig 2), and the total number of clones contained in the *C. horii* library was 47800.

To check for the presence of the inserts, 50 colonies of XL1-Blue MR were randomly selected from LB-ampicillin plates. The cosmids were isolated, and then subjected to electrophoresis on a 0.7 % agarose gel. Although the exact sizes of cosmids harboring the inserts were not measured by the agarose gel, the cosmids were detected in 20 clones, as shown in Fig 3. The remaining 30 clones examined

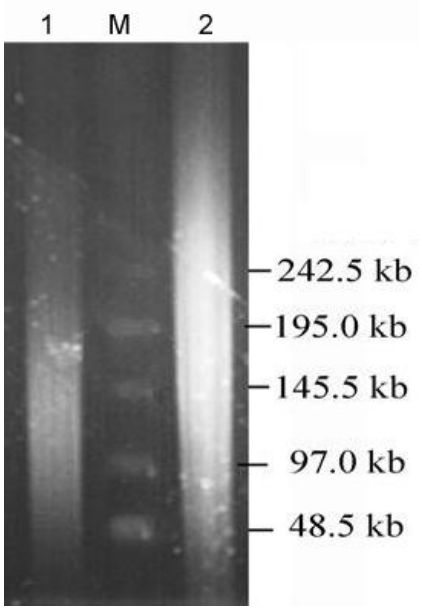

Fig. 1 – Pulsed-field gel electrophoresis (PFGE) pattern of genomic DNA from *Colletotrichum horii* performed at 148C in a 0.5 % agarose gel. Lane 1. Purified genomic DNA. Lane 2. Purified genomic DNA by picking out with a capillary. Lane M, lambda ladder PFG marker (New England Biolabs, USA)

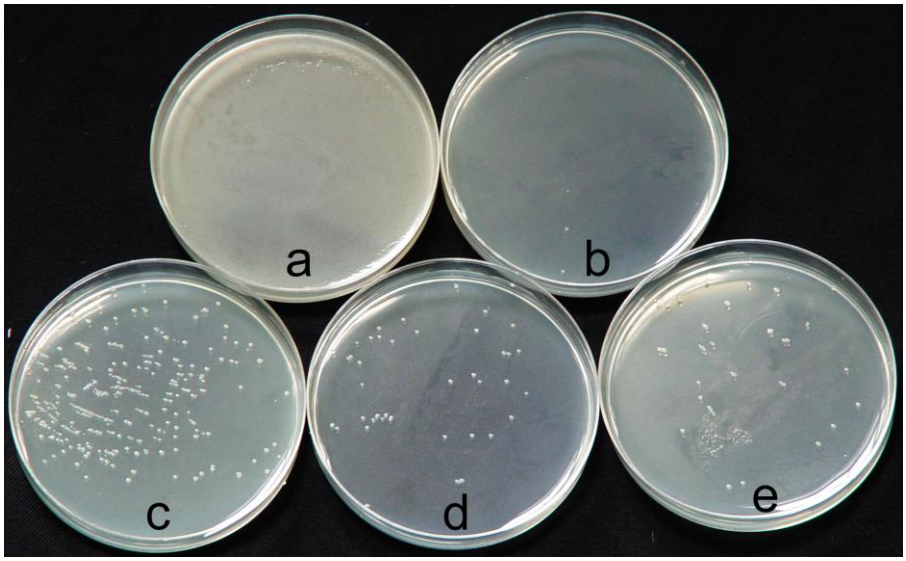

Fig. 2 – The colonies of bacterium strain XL1-Blue MR containing the packaged cosmids on LB-ampicillin selection plates with a serial dilution. (a) Positive control (VCS 257strain with Lamda DNA); (b) Negative control (no DNA); (c) A plate with a 1: 10 dilution. d. A plate with a 1: 50 dilution; (d) A plate with 1: 100 dilution.

were also of the same size (data not shown). The result shows the presence of inserts of about 40.5 kb ligated in the 7.9 kb of cosmid vectors and clones lacking inserts were not detected.

The cosmid DNA of 50 clones were digested by *Not*I and analysis of gel electrophoresis showed the insert size of 50 randomly picked clones ranged between 30 and 42 kb with an average of 34.8 kb. The products of digested cosmids were detected in 16 clones, as showing Fig 4.

Similarly, the cosmid DNA of 50 clones was digested by *Eco*RI and analysis of

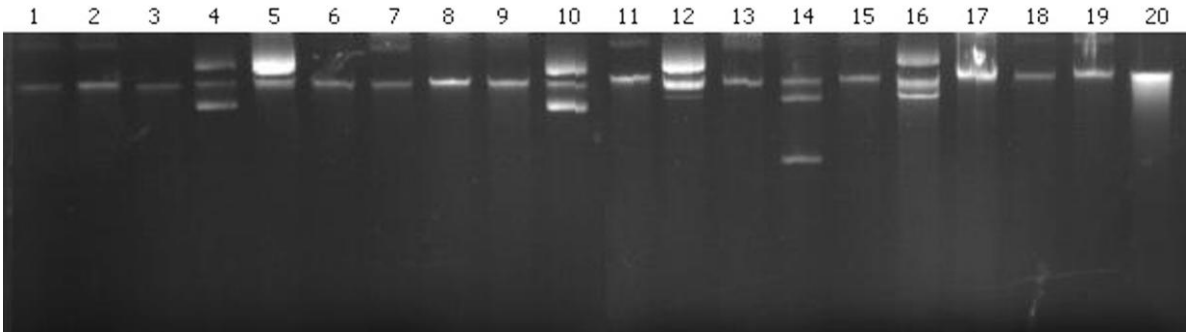

Fig. 3 – Insert check for the packaged cosmids. Lanes 1–20, cosmids purified from the infected XL1-Blue MR colonies. Cosmids were separated on a 0.7 % agarose gel

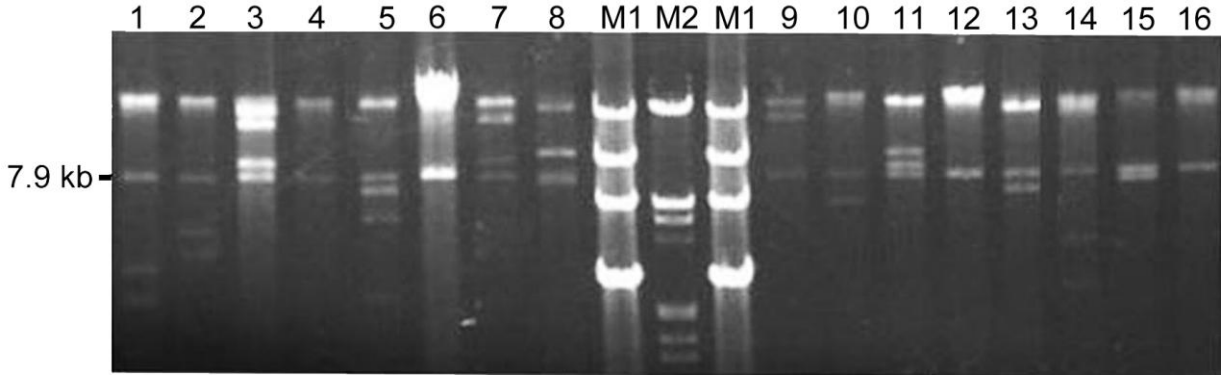

Fig. 4 – Insert check for the packaged cosmids with a *Not*I digestion. Lanes 1–16, cosmids purified from the infected XL1-Blue MR colonies. (M1) DNA size Marker(15; 8; 5 and 2.5 kb, Takara, Japan); (M2) Lamda DNA / *Eco*RI & *Hin*dIII (21.2, 5.1, 4.2, 3.5, 2.0, 1.6 and 1.4 kb, MBI, Ferments)

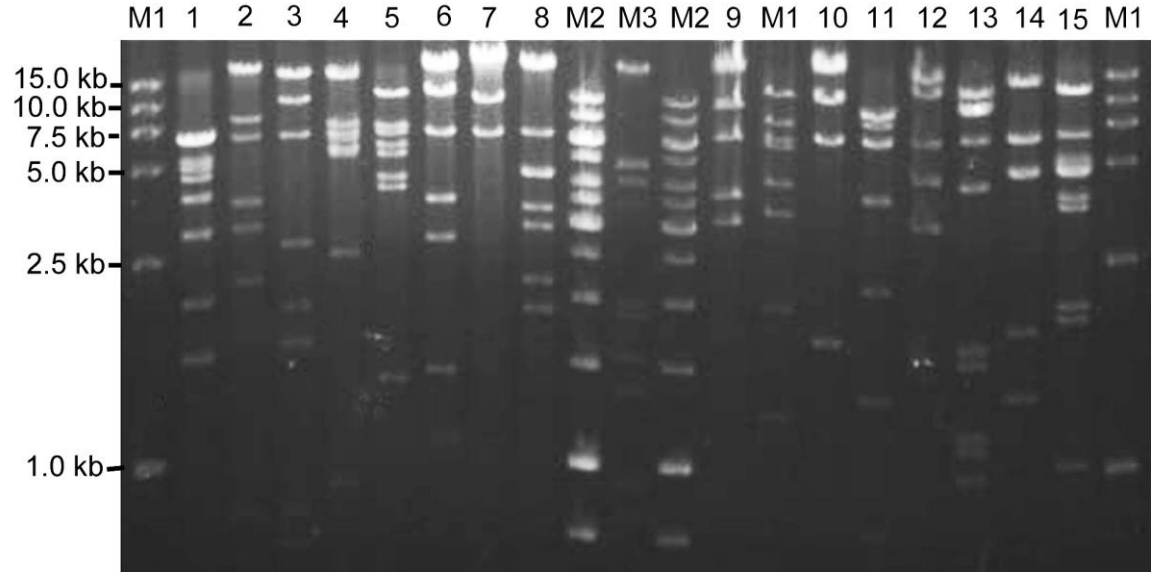

Fig. 5 – Insert check for the packaged cosmids with a *Eco*RI digestion. Lanes 1–15, cosmids purified from the infected XL1-Blue MR colonies. (M1) DNA size Marker (Takara, Japan)； (M2) DNA size Marker (10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.5, 1.0, .07, 0.5 and 0.3kb; Axygen, USA)； (M3) lamda DNA/*Eco*RI & *Hin*dIII (21.2, 5.1, 4.2, 3.5, 2.0, 1.6 and 1.4 kb, MBI, Ferments

Table 1 The relationship of average inserts size and necessary number of colonies

Genome size(bp)	necessary number of colonies(N)	Average insert size(bp)	*P**
5×10^7	6614	34.8×10^3	0.99
5×10^7	6871	33.5×10^3	0.99

**P* is the probability to find any given DNA sequence and was calculated by using an equation from Clarke & Carbon (1976); N = ln (1-P)/ln (1-f), where *f* is the fractional proportion of the genome in a single recombinant: f = average insert size (bp)/genome size (bp).

gel electrophoresis indicated that the insert size of 50 randomly picked clones ranged between 24-42 kb with an average of 33.5 kb (Fig 5).

Characterization of the library

The exact genome size of *C. horii* is unknown so far but the genome DNA content of *C. graminicola* had been shown to be 4.8-5.0×10^7 bp (Randhir & Hanau, 1997). Therefore, the genome size of *C. horii* can be estimated within 5.0×10^7 bp. An equation from Clarke & Carbon (1976) was used to calculate necessary number of colonies or the number of primary recombinants for the desired probability to find any given DNA sequence represented in the library (Table 1). Based on the number of primary recombinants (number of clones), the genome coverage of this library was approximately 6.96 genome equivalents or 7.22.

The packaged cosmids were amplified in XL1-Blue MR host cells as recommended by the manufacturer and the colonies were counted and the titer (cfu/mL) was calculated as described above. The result of cosmid library amplification showed that the titer of packaged cosmids was 4.6×10^8 cfu/mL. After amplifying cosmid library, the cell suspension with 15% glycerol and 100 μg/ml ampicillin was store in 1.5mL microfuge tubes at -80 °C.

After the freeze/thaw cycle for three times, the result for titering cosmid library showed that the titer of packaged the titer of packaged cosmids was 4.0×10^8 cfu/mL.

Discussion

Species of *Colletotrichum* use diverse strategies for invading host tissue, ranging from intracellular hemibiotrophy to subcuticular intramural necrotrophy. In addition, these pathogens develop a series of specialized infection structures, including germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae. *Colletotrichum* species provide excellent models for studying the molecular basis of infection structure differentiation and fungal–plant interactions (Perfec et al.1999). Cytological studies clearly show that the infection process and intracellular infection structures of *C. horii* are different from that of *C. gloeosporioides* (Sutton 1992) and similar to hemibiotrophic species of *Colletotrichum* (Perfec et al.1999), but more closely related to that of *C. lindemuthianum* on bean (O'Connel et al. 1985). How-ever, the molecular mechanisms of interaction between pathogen and host are not well understood. The genomic libraries were used wisely as an important means for the target gene cloning in *Colletotrichum* (Huser et al. 2009, Kim et al. 2000) while construction of the genomic library of *C. horii* provides a important approaches for isolation of genes related to molecular events.

In construction of the genomic library, it is important to prepare larger genomic DNA. By obtaining mycelia producing from conidial germination and removing smaller DNA fragments with a capillary, we established successfully a method to isolate DNA of high molecular weight with sufficient purity and to construct a cosmid library. To our knowledge, this is the first cosmid library constructed for a persimmon anthracnose pathogen. The DNA was easily digestible and partial digestion conditions with *Sau* 3AI that yielded the majority of DNA fragments in the 30-42 kb range were established as well. The library of *C. horii* reported here was shown to be representative for the entire genome with a calculated 99 % probability to find any given

DNA sequence. Because the amount of insert DNA included in the ligation reaction is proportional to the eventual number of clones in the library, after amplifying cosmid library, our library is to obtain more concentrated insert DNA and to construct a library having many more clones. Although southern blot analysis data is not showed for determining the kind of insert DNA, these will be indicated detailly in another paper, while in this study, we put more emphasis on the construction and characterization of a genomic library of *C. horii* . Much work associated with cloning of pathogenesis-related genes is being conducted using this library, which will be a valuable source for the isolation of genes from the fungal *C. horii* .

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