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RNA-seq for comparative transcript profiling of *Phytophthora capsici* during its interaction with *Arabidopsis thaliana*

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ABSTRACT

Phytophthora capsici, a highly dynamic and destructive oomycete pathogen, causes devastating diseases on a wide range of plants worldwide. However, the detailed molecular mechanisms of pathogenicity is still largely unclear. In this study, three different mRNA pool libraries were constructed from its developmental stage, early or late infection stage of the model plant *Arabidopsis thaliana*, and then were investigated by the RNA-Seq approach. The results demonstrated that 1456 novel transcripts that had not linked to any annotated gene were identified, and 296 genes were found to undergo alternative splicing. Comparative analysis of three different libraries further showed that distinct transcriptional changes of pathogenicity genes were found. A large number of genes containing cell wall degrading enzymes, major facilitator superfamily genes and cytochrome P450 genes were highly induced during infection. In addition, several types of well-known effectors including RxLR, CRN, Elicitin and NLP proteins also showed high transcript abundances during infection. The transcriptional levels of six effector genes during the infection process were further validated by qRT-PCR. Collectively, this study provides a basic understanding of pathogenic mechanisms of *P. capsici* during the interaction with plants.

1. Introduction

Oomycetes are a group of eukaryotic microorganisms that have colonized many ecological niches, and more than 60% of the known oomycete species are parasitic on a large number of agriculturally important crops worldwide [1]. Oomycetes are fungus-like organisms that have close relationship with algae and are classified into the kingdom of *Stramenopila* [2,3]. The pathogenicity of oomycetes evolves independently from pathogens in other lineages such as fungi, and a variety of biochemical tests show that oomycetes are not inhibited by many of the chemicals used to control fungi. Until now, the battle with oomycetes-related diseases has been initially unraveled, and news related to plant diseases caused by oomycetes tend to capture the interest of the general public.

The genus of *Phytophthora* contains a group of destructive plant pathogens, which cause devastating effect on a wide range of important plants [4,5]. For example, *P. infestans* triggered the Irish potato famine in the mid-nineteenth century and remains a severe problem worldwide, and *P. sojae* causes millions of dollars of losses in soybean industry every year [6]. *P. capsici* is a highly destructive pathogen that causes severe infection to a number of important vegetables such as pepper, tomato, eggplant and lima beans [7]. *P. capsici* is treated as a model to understand broad-host-range oomycetes and the basic mechanisms of *Phytophthora* virulence. The correlate diseases caused by *P. capsici* in vegetable planting areas are difficult to be controlled using routine methods. Great economic losses can be induced by *P. capsici* during planting and transportation of vegetables [7]. It is urgent to understand how this pathogen manipulates the host and promotes pathogen growth and reproduction. Recently, more and more tools and resources have been developed to study this devastating pathogen, including the release of genomic sequences [8,9].

Phytophthora species are regarded as hemibiotrophs that are biotrophic initially but switch to necrotrophic later. In the initial biotrophic phase, haustoria are formed to penetrate to the plant cell walls and interface with the plant membrane [10], and this phase is crucial for disease establishment, followed by rapid intercellular growth and colonization come up, leading to plant cell death. Some studies have reported that these different phages need specific expression of many genes [11]. Plants are attacked by many pathogens, and defend themselves via pattern recognition receptors that recognize pathogen-associated molecular patterns and trigger effective immune responses. To counter plant defenses, pathogens usually secrete a large number of effectors into the host apoplast (extracellular effectors) or cell cytoplasm (intracellular effectors), followed by modifying cellular targets to

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suppress plant defense and enable parasitic infection and reproduction [12].

The development of durable strategies to control plant diseases is therefore a major challenge when striving for successful plant protection. To achieve this, increased knowledge on the mechanisms underlying plant-pathogen interactions would be greatly helpful. Hence, it is necessary to investigate the molecular processes regulating the life cycle stages of *Phytophthora* for effective identification of pathogenesis and improving of control strategies. One strategy for exploring these processes is to investigate pathogen and plant gene-expression patterns during the infection process. Novel high-throughput deep sequencing technologies have led to an increasing growth in the analysis of infection-associated genes of pathogens. This will help the identification of important transcriptional changes that occur in the pathogen during its infection.

Since the release of whole genome sequences of some important *Phytophthora* species, as well as a massive explosion of transcriptome data, we have greatly facilitated the understanding of the pathogenic mechanisms underlying *Phytophthora*. By mining numerous data involving the genome and transcriptome, bioinformatics can provide research clues and better candidates to improve the efficiency of the functional analyses of pathogenicity factors and candidate effectors [13]. Recent studies of *Phytophthora* pathogens have focused on secreted effectors because of increasing evidence that these proteins modulate the plant defense responses [14–16]. The availability of a high-quality reference genome has accelerated research aiming to study various aspects of *P. capsici* biology, including the pathogenesis and effector evolution [9,17].

In the present study, we performed RNA-Seq approach to investigate the dynamic changes of *P. capsici* transcriptome during the interaction with *Arabidopsis thaliana*. Our analysis revealed numerous novel transcripts and alternative splicing events, which could improve the current genome annotation of *P. capsici*. In addition, we identified a large number of differentially expressed genes involved in the infection process, which would help us understand more about pathogenic mechanisms of *P. capsici* during its interaction with plants.

2. Materials and methods

2.1. P. capsici and plant materials

A *P. capsici* isolate (Pc35) was isolated from diseased crop in China, preserved in liquid nitrogen and then maintained on 10% V8 agar media at 25 °C in the dark. The *P. capsici* mycelia were cultivated in 10% V8 liquid medium at 25 °C in the dark for 3 days and were collected in liquid nitrogen for RNA isolation. Sporangium was induced by repeatedly washing mycelial mat with sterile distilled water at 25 °C in the dark until sporangia formed abundantly. Zoospores were released by placing the zoosporangial mycelial mat into 10 ml of sterile distilled water at 5 °C for 30 min, and then, at 25 °C for 40 min. The zoospores were concentrated by centrifugation at 2000 rpm at 0 °C and were then preserved in liquid nitrogen for RNA isolation. Cysts were produced by vortexing the zoospores suspension at room temperature for 90 s and wait for 30 min then collected by centrifugation at 2000 rpm at 0 °C and were preserved in liquid nitrogen for RNA isolation. Germinating cysts were obtained by cultivating cysts with 5% V8 liquid medium at 25 °C, 150 rpm for 1 h and were then collected by centrifugation at 2000 rpm at 0 °C.

The mycelial infection was performed following the previous papers [18,19]. Briefly, a mycelial mat was washed with sterile distilled water and then sandwiched between upper surfaces of two *A. thaliana* leaves at 25 °C for 1, 2, 4, and 8 h post infection (hpi), respectively. The mycelial mat was carefully peeled from the leaves and preserved in liquid nitrogen. For the sample at 48 hpi, the *A. thaliana* leaves were inserted with mycelia, followed by excised together with the mycelia and then were preserved in liquid nitrogen. *A. thaliana* seeds were grown in the greenhouse at 22 °C, with a photoperiod of 16 h, supplemented by artificial light. *A. thaliana* leaves (6–8 weeks old) were treated with 0.05% vol/vol solution of Tween 20 to improve wetting and placed upside-down in humid plastic trays in the greenhouse with the same settings.

2.2. Library preparation and sequencing

The RNAs of mycelium, sporangium, zoospore, cyst and germinating cyst were mixed with equivalent concentration as the asexual development stage (DV). RNAs of mycelial infection for 1, 2, 4 hpi were mixed with equivalent concentration as the early infection stage (EI). RNAs of 8 and 48 hpi samples were mixed with equivalent concentration as the late infection stage (LI). RNA was isolated from frozen tissue (Total RNA purification system; Invitrogen, Carlsbad, CA, U.S.A.), and then sequenced on an Illumina HiSeq 2500. The produced reads were paired-end 2 × 150 bp.

2.3. Transcriptome analysis

The *P. capsici* genome and gene information were downloaded from JGI (http://genome.jgi.doe.gov/Phyca11/Phyca11.home.html). After removing reads of low quality (reads containing Ns > 5), the remaining reads were aligned to *P. capsici* reference genome using Tophat, allowing up to 2 base mismatches [20]. Minimum and maximum intron lengths were fixed at 50 and 5000 bp, respectively. Moreover, reads mapping to more than one genomic position were excluded, and only the uniquely mapped reads were retained for subsequent processing. The novel transcripts were predicted in the intergenic regions by Cufflinks tool, and novel transcripts with length < 90 bp were excluded. TransDecoder software was performed to identify potential coding regions within the novel transcripts.

Tophat tool was performed to predict splice junctions. We concentrated on gapped reads that aligned across introns and were from exon-exon junctions. To reduce false positives because of alignment artifacts, we only analyzed gapped reads that aligned with at least 5 bp on either side of the intron. Novel splice junctions were generated by comparing with known splice junctions in the *P. capsici* annotations. MATS tool [21] was applied to identify potential alternative splicing (AS) events in *P. capsici*. The identified junction sequences were used to analyze four different AS types including retained intron, alternative 5′ splice site, alternative 3′ splice site and skipped exon. To validate the accuracy of the predicted AS events, two genes (Pc547874 and Pc504456) were amplified by RT-PCR. Specific primers (Supplementary Table 1) were designed in the flanking regions of the AS sites in the two genes, and the *P. capsici actin* gene was used as a control.

To compare the gene expression level in each library, the transcript levels of each gene were calculated and normalized to reads per kilobase of exon region per million mapped reads (RPKM). To filter out weakly expressed genes, only genes with RPKM larger than one were retained in the further analysis. Differentially expressed genes were identified using the GFOLD algorithm, which was more biologically meaningful for single replicate experiments [22]. Genes with at least four fold change (GFOLD > 1 or < −1) were considered differentially expressed between each pair of two libraries.

2.4. qRT-PCR analysis

To validate the RNA-Seq results, qRT-PCR was applied for six effector encoding genes (Supplementary Table 1) which were up-regulated by more than four fold based on RNA-Seq analysis. First strand cDNA was synthesized using an iScript cDNA Synthesis kit (Takara Bio DRR036A). The gene encoding tubulin was selected as the internal control to normalize the expression data. Quantitative real-time PCR thermal cycler conditions and reaction mixtures were performed according to the manufacturer's instructions (SYBR® Premix Ex Taq™). To ensure the accuracy, at least three biologically independent