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# Transcriptome profiling to identify genes involved in pathogenicity of *Valsa mali* on apple tree

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ABSTRACT

Apple Valsa canker, caused by the fungus *Valsa mali* (*Vm*), is one of the most destructive diseases of apple in China. A better understanding of this host–pathogen interaction is urgently needed to improve management strategies. In the current study we sequenced the transcriptomes of *Vm* during infection of apple bark and mycelium grown in axenic culture using Illumina RNA-Seq technology. We identified 437 genes that were differentially expressed during fungal infection compared to fungal mycelium grown in axenic culture. One hundred and thirty nine of these 437 genes showed more than two fold higher transcript abundance during infection. GO and KEGG enrichment analyses of the up-regulated genes suggest prevalence of genes associated with pectin catabolic, hydrolase activity and secondary metabolite biosynthesis during fungal infection. Some of the up-regulated genes associated with loss of pathogenicity and reduced virulence annotated by host–pathogen interaction databases may also be involved in cell wall hydrolysis and secondary metabolite transport, including a glycoside hydrolase family 28 protein, a peptidase and two major facilitator superfamily proteins. This highlights the importance of secondary metabolites and cell wall hydrolases during establishment of apple Valsa canker. Functional verification of the genes involved in pathogenicity of *Vm* will allow us to better understand how the fungus interferes with the host machinery and assists in apple canker establishment.



## 1. Introduction

*Valsa mali* (*Vm*), the causal agent of apple Valsa canker (Wang et al., 2014), is one of the most destructive diseases on apple in eastern Asia (Gao and Liu, 1995; Sakuma, 1990), especially in China (Cao et al., 2009; Wang et al., 2005), Japan (Abe et al., 2011) and Korea (Uhm, 2006). Previous studies revealed that *Vm* exhibits only weak virulence, and that wounds including frostbites, sunscalds, pruning ends or other mechanical injuries substantially favor infection (Adams et al., 2006; Biggs, 1989; Kepley and Jacobi, 2000; Willison, 1936). Once inside the host tissue the fungus can induce tissue maceration and cell death. A cytochemical study revealed that pectinases secreted by *Vm* must play an important role during the colonization of apple (Ke et al., 2013). However, there is still no molecular information about the genes that are potentially associated with virulence. Analyses of the genomes of necrotrophic plant pathogenic fungi such as *Pyrenophora teres* and *Stagonospora nodorum* revealed an abundance of genes encoding products that are intuitively associated with the necrotrophic life style, including protein and carbohydrate hydrolases, efflux pumps, cytochrome P450s, nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) (Ellwood et al., 2010; Hane et al., 2007; Lévesque et al., 2010). Protein and carbohydrate hydrolases are related to the degradation of plant cuticle and cell wall, while cytochrome P450s, NRPSs and PKSs are involved in secondary metabolite and cyclic peptide biosynthesis. These are extruded into the host with the help of efflux pumps (Schmidt and Panstruga, 2011). However, in fungi genes associated with secondary metabolite biosynthesis are often silent under standard laboratory conditions (reviewed in Brakhage and Schroeckh, 2011). The current strategy is to attempt to simulate the fungus by plant–pathogen interaction.

As recent studies suggest, necrotrophic fungal pathogens can subtly manipulate the host during infection in a manner similar to biotrophic pathogens through effectors to initiate disease (Oliver and Solomon, 2010; Hammond-Kosack and Rudd, 2008). Secreted proteins and other molecules, collectively known as effectors, are widely accepted as critical for a mechanistic understanding

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of the processes underlying host colonization and pathogenicity (reviewed in Hogenhout et al., 2009). Many protein factors are involved in the process of infection and the establishment of a parasitic fungal–plant interaction, such as cell wall degrading enzymes (Friesen et al., 2008). Even so, low molecular weight phytotoxins often play a key role in infection and virulence. Typically, such fungal secondary metabolites alone reproduce some or even all of the symptoms of the disease caused by the fungal producer organisms (Oliver and Solomon, 2008). In the past years, many analytical methods for elucidation of metabolite structures have been improved and research in this field has been propelled based on the availability of genome sequences of fungal pathogens and bioinformatics (Dean et al., 2005; Kämper et al., 2006).

The current absence of a sequenced genome prevents many valuable experimental approaches from being applied to *Vm*, including determination of gene expression patterns after inoculation of apple twigs, comparison of gene content to other phytopathogenic fungi, and reverse genetic manipulation to determine gene function. Despite the absence of a fully sequenced and assembled genome, many of these experiments could be pursued after sequencing the transcriptome. Short read sequencing technologies such as Solexa/Illumina (Illumina), 454 (Roche), and SOLiD (ABI) platforms have made it possible to perform *de novo* transcriptome sequencing (Hudson, 2008; Wang et al., 2009). Therefore, in the current study, 'next generation' sequencing technology offered by Illumina PE-SBC (Shanghai Biotechnology Corporation) was used to analyze the transcriptomes of *Vm* during infection on apple twigs, and mycelium of *Vm* grown on PDA (potato dextrose agar). In this report we present a functional classification based on assignments to publicly available datasets, gene expression profiles, and potential pathogenicity determinant predictions conducted to reveal virulence or pathogenicity genes of *Vm*.

## 2. Materials and methods

### 2.1. Fungal strain, culture conditions and inoculation

*Valsa mali* (*Vm*) isolate 03-8, a single conidium pure culture isolated from apple bark with Valsa canker, was obtained from the Laboratory of Integrated Management of Plant Diseases in College of Plant Protection, Northwest A&F University. The isolate was cultured on potato dextrose agar (PDA) covered with a layer of cellophane. Plates were incubated at 25 °C until the diameter of the mycelial mat reached 5–6 cm (3–4 days). 0.2 g mycelium was harvested from each plate, placed in sterile 2 mL centrifuge tubes, and frozen in liquid nitrogen. Three independent mycelial samples were collected for qRT-PCR analysis and one of them was used for RNA-sequencing.

For transcriptome analysis of *Vm* during infection, biennial intact apple twigs of *Malus domestica* borkh. cv. 'Fuji' from the green house were collected and inoculated according to Zang et al. (2007). Twigs were cut into 25 cm long segments and washed with tap water, immersed in 1% sodium hypochlorite for 10 min, then rinsed with sterile water three times. The ends of the twigs were sealed with wax. Each twig segment was subjected to four wounds with a flat iron (5 mm diam). Wounds were 5 cm apart. Twigs were placed horizontally in a plastic box. A 5-mm circular agar plug with mycelium of *Vm* was inoculated on each scorch site. Then the box was immediately covered with a vinyl film to retain humidity. For three infected twigs, 0.2 g of each of three lesions were collected five days post inoculation (dpi) at 25 °C, frozen in liquid nitrogen, and stored at −80 °C. Three replicates of infected tissues were used for qRT-PCR and one of them was used for RNA-sequencing.

### 2.2. RNA extraction and sequencing

Total RNA from each of the collected samples was isolated using the RNeasy Micro kit (Qiagen, Shenzhen, PRC) according to the manufacturer's protocol. The integrity and quantity of isolated RNA were assessed on a Bioanalyser. Six μg RNA from each sample was used for RNA library preparation using the TruSeq RNA sample Prep Kit (Illumina) as per the manufacturere's instruction. Libraries were quantified using a Qubit® 2.0 Fluorometer (Life technologies). Ten pM of the prepared library was loaded onto a flowcell for cluster generation following the cBot User Guide (Illumina). Sequencing was performed with paired-end $2 \times 100$ nt multiplex procedure on an Illumina HiSeq 2000 following the manufacturer's instructions (Illumina).

### 2.3. Raw reads cleaning, assembly and sequence annotation

Raw sequencing data was filtered using a Perl script DynamicTrim.pl (Cox et al., 2010), removing adaptor sequences, empty reads and low quality sequences and short reads (<25 bp). Resulting reads were stored in FASTQ format. To remove apple specific reads, reads from infected apple bark were mapped onto the apple genome (Velasco et al., 2010) using the program tophat (Kim et al., 2013) with default settings. Unmapped reads were considered to originate from *Vm*. *De novo* assembly of high quality reads was performed using Trinity (Grabherr et al., 2011). After assembly, contigs (>300 bp) were used for gene prediction by transcripts_to_best_scoring_ORFs (ORF: Open reading frame) from the Trinity package (Version trinityrnaseq-r2013-02-25).

Predicted genes of *Vm* were searched against Genbank's NR databases (*E*-value <1e−3) to assign associated GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) annotations using Blast2GO (Götz et al., 2008). PHI-base (Winnenburg et al., 2006), was searched for different phenotypic categories like loss of pathogenicity, reduced virulence, lethality, increased virulence, etc. using BLASTp (*E*-value <1e−5). GO enrichment analysis (Fisher's Exact test, Blüthgen et al., 2005) was carried out by Blast2GO (Götz et al., 2008), and KEGG enrichment analysis was carried out using the hypergeometric test (Subramanian et al., 2005).

### 2.4. Gene expression profiling in mycelium grown in vitro and during infection

Gene expression levels can be estimated from Illumina sequencing based on the number of raw reads (Mortazavi et al., 2008). Reads from both samples were mapped back to the assembled transcripts and expression abundance calculated using the program run_RSEM_align_n_estimate from the trinity package. Expression levels are presented with FPKM (fragments per kilobase of exon model per million mapped reads) using the program Cuffdiff (Cufflinks, V2.1.1) according to Trapnell et al. (2010). Statistical comparison was performed using the *P*-value <0.05 and fold-change $\geqslant 2$ or $\leqslant -2$ as threshold.

### 2.5. Quantitative reverse transcription-PCR (qRT-PCR)

To confirm results from the differential expression analysis, fifteen genes with a fold-change greater than 0 were randomly selected for qPCR verification. Reverse transcription was performed using an ABI 7500 Real-Time PCR System and 7500 System Software (Applied Biosystems, USA) using a SYBR Green based PCR assay. The reaction was performed in a total reaction mix including cDNA (1 μL), PCR primers (10 μM, 0.4 μL), and SYBR Premix ExTaq ($2 \times 5$ μL; TaKaRa Bio Inc.) in a total volume of 25 μL. PCR protocol was as followed: 95 °C for 1 min, 40 cycles of 95 °C for 10 s, 60 °C for 10 s, then 72 °C for 40 s. Finally, dissociation curves were

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