Physiological and Molecular Plant Pathology 91 (2015) 1–10

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Characterization of the secretome of *Plasmopara viticola* by *de novo* transcriptome analysis

Ling Yin [a, b, 1], Xinlong Li [a, 1], Jiang Xiang [a], Junjie Qu [c], Yali Zhang [a], Ian B. Dry [b, **], Jiang Lu [a, *]

[a] China Agricultural University, College of Food Science and Nutritional Engineering, Beijing 100083, PR China
[b] CSIRO Agriculture, Wine Innovation West Building, Waite Campus, Hartley Grove, Urrbrae, SA 5064, Australia
[c] Guangxi Academy of Agricultural Sciences, Guangxi Crop Genetic Improvement and Biotechnology Key Lab, Nanning 530007, PR China

ARTICLE INFO

Article history:
Accepted 5 May 2015
Available online 14 May 2015

Keywords:
Downy mildew
Plasmopara viticola
Secretome
Effector
RXLR

ABSTRACT

Plasmopara viticola is an oomycete pathogen that causes downy mildew, one of the most devastating diseases of grapevine. Currently, the molecular basis of the interaction between this pathogen and the grapevine host is not well understood. To explore the genes involved in *P. viticola* pathogenicity we performed RNA-Seq analysis of cDNAs derived from downy mildew-infected grapevine leaves for three different *P. viticola* isolates; two from China (JL-7-2, ZJ-1-1) and one from Australia (CSIRO-L-2). Approximately 30,000 unigenes were predicted for each of the three isolates and from these over 500 potential secreted proteins were identified. Using three prediction methods, a total of 51 PvRXLR effectors were identified to be present in the secretome, with at least 26 shared by two or more *P. viticola* isolates. Expression profiling, based on RNA-Seq data, indicated that PvRXLRs showed three different expression patterns during infection. Transient expression of selected PvRXLRs in *Nicotiana benthamiana* demonstrated that all were capable of suppressing programmed cell death triggered by the mouse BAX protein or the PAMP INF1. Additionally, BLASTP and Hidden Markov Model (HMM) searches identified 10 predicted proteins belonging to the CRN (Crinkler) group of oomycete effectors. Pfam domain analysis of the secretome also identified a diverse range of putative apoplastic effectors, the major groups being glycosyl hydrolases, peptidases and protease-inhibitors. This study provides the first detailed analysis of the secretome of grapevine downy mildew and its encoded effector arsenal.

Introduction

The downy mildews are well-known oomycete pathogens with a narrow host range. Grape downy mildew, caused by *Plasmopara viticola* ([Berk. et Curt.] Berl. et de Toni), is a highly destructive disease worldwide [1]. Most cultivated winegrapes are derived from the Eurasian grape species, *Vitis vinifera*, and have little resistance to this oomycete pathogen which originated from North America. Repeated applications of fungicides are currently the most effective method of protection, but this is costly for grapegrowers and is potentially harmful to the environment. The introduction of resistance (*R*) genes from wild grape species into susceptible *V. vinifera* cultivars would be of significant economic and environmental benefit [2]. However, the diversity and evolutionary potential of the pathogen populations pose a challenge to breeding strategies for durable resistance. In order to improve breeding (strategies) efficiency for the disease resistance of grapevines, it is essential to understand the pathogenesis mechanisms by which a pathogen can break down plant defenses.

P. viticola is an obligate biotrophic pathogen that derives all of its nutrition from living cells via globose haustoria. Leaf infection starts with a sporangium landing on the plant's epidermis. In the presence of moisture, biflagellate zoospores are released from the sporangia and swim towards a stomate where they encyst. The zoospores then germinate and penetrate through a stoma by the means of a germ tube which forms an infection vesicle within the substomatal cavity [3]. Primary hyphae emerge from the infection vesicle and form a mycelium that spreads into the spongy parenchyma, penetrating cells with haustoria. After 5–21 days, and in the

* Corresponding author.
** Corresponding author.
E-mail addresses: yin.ling.1985@gmail.com (L. Yin), 547722872@qq.com (X. Li), haoyan7371@gmail.com (J. Xiang), junjie.qu.2010@gmail.com (J. Qu), olivia.yl.zhang@gmail.com (Y. Zhang), ian.dry@csiro.au (I.B. Dry), j.lu.cau@gmail.com (J. Lu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.pmpp.2015.05.002
0885-5765/

presence of high humidity, tree-like sporangiophores, bearing white, lemon-shaped sporangia, are produced from the mycelium within the host tissue and emerge through the stomates of the infected tissue [1,4].

The interaction between pathogens and host plants is a dynamic evolutionary battle which can be described as a 'zigzag' model [5]. To prevent infection by biotrophic pathogens, such as downy mildew, plants have evolved a dual layered immune system to defend themselves. These two layers are known as Pathogen Associated Molecular Patterns (PAMP)-triggered immunity (PTI) and Effector-triggered immunity (ETI). PTI is activated by the recognition of pathogen-specific molecules by pattern recognition receptors in the plasma membrane [6]. Pathogens which are able to successfully invade host cells are able to suppress PTI by the secretion of effector proteins which target components of the PTI defense pathway, as well as manipulate host metabolism to facilitate the biotrophic interaction. ETI involves recognition of specific effectors either directly or indirectly by R proteins which then induce a range of defense responses including PCD [7]. Therefore the identification of the complement of secreted effectors of a pathogen is critical in understanding the mechanisms of both pathogen infection and plant resistance.

Over the last few years, studies on oomycete effectors have made great progress. Genome sequencing of a number of hemibiotrophic *Phytophthora* pathogen species [8,9] and the biotrophic Arabidopsis downy mildew pathogen *Hyaloperonospora arabidopsidis* [10] has revealed the presence of hundreds of genes within the genomes of these pathogens that encode secreted proteins that could potentially act as effectors [10–12].

The best studied are the RXLR effectors, named for a conserved N-terminal amino acid motif consisting of arginine, any amino acid, leucine, arginine. Many of these RXLR effectors also have a second motif EER at varying distances C-terminal to the RXLR motif. The RXLR and EER motifs are required for entry of the effector into the host cell [13,14]. The RXLR effectors are also particularly important in ETI-mediated resistance as demonstrated by the fact that most of the cloned avirulence (Avr) effectors (references summarized in Ref. [12]) contain an RXLR motif. Some variants of the RXLR motif have also been identified, including the QXLR motif found in host-translocating effectors of cucurbit downy mildew [15].

The second major class of secreted effectors that have been shown to be produced by oomycete pathogens are the crinkling- and necrosis-inducing proteins (CRN) or Crinklers [12]. The CRN protein family encodes proteins feature a conserved N-terminal LXLFLAK motif required for host cell entry connected to diverse C-terminal effector domains [16,17]. CRN proteins appear to be ubiquitous in plant pathogenic oomycetes, whereas the RXLR effectors are only found in the Peronosporales (*Phytophthora* species and downy mildews) and Albuginales (*Albugo* and other white rusts) suggesting that RXLR effectors may have evolved coincident with the emergence of haustoria [18].

Currently, knowledge about the interaction between *P. viticola* effector genes and their targets in grapevine is relatively poor. A small number of candidate effector genes from *P. viticola* have previously been reported [19], but these were identified in a cDNA library constructed from *in vitro* germinated zoospores, rather than from *P. viticola* during the infection process. Furthermore, with the recent cloning of first grapevine downy mildew resistance gene *MrRPV1* [2], there is particular interest in trying to identify the Avr effector(s) secreted by *P. viticola* that initiate the ETI response mediated by MrRPV1. With the advent of highly sensitive next-generation sequencing technologies it is now possible to characterize the secretome of a biotrophic pathogen by transcriptome sequencing of infected plant tissues, followed by *in silico* removal of the host transcripts. This has one important advantage over genome sequencing in that it identifies which genes are expressed during the infection process. Here we report a *de novo* transcriptome assembly of *P. viticola* by RNA-Seq analysis of cDNAs derived from infected grapevine leaves at different time points after inoculation and the use of *in silico* approaches to predict and describe the secretome of this grape pathogen.

Materials and methods

P. viticola isolates, plant material and sample collection

Three *P. viticola* isolates (CSIRO-L-2 , JL-7-2 and ZJ-1-1) used in this study were collected from different grapevine cultivars and purified using single-sporangiophore inoculation. The Australian isolate CSIRO-L-2 was collected from an experimental vineyard on the Waite Campus, Adelaide, Australia, and then maintained on detached leaves of *V. vinifera* cv. Cabernet Sauvignon under controlled greenhouse conditions (22 °C under a 16 h light/8 h dark cycle). The two Chinese isolates JL-7-2 and ZJ-1-1 were isolated from *Vitis riparia* Michx. cv. Beda and *Vitis amurensis* cv. Shuanghong grapevines respectively growing in Northeast China. They were maintained by subsequent inoculations on *V. vinifera* cv. Thompson Seedless plants under the same conditions as above.

Discs (1 cm in diameter) were cut from duplicate leaves (nodes 4–7), inoculated with 100–200 μl droplets *P. viticola* sporangia suspension in water to cover almost the whole disc (approximately 5×10^6 sporangia/ml) and incubated in sealed petri dishes at 22 °C under a 16 h light/8 h dark cycle for 6 days. For the CSIRO-L-2 isolate, inoculated *V. vinifera* cv. Cabernet Sauvignon discs were collected at 12, 24, 48, 72 and 96 h post-inoculation (hpi). Alternatively, leaves of *V. vinifera* cv. Cabernet Sauvignon plants were spray-inoculated in the glasshouse, bagged overnight and left for 10 days until oil-spot symptoms were observed. Infected tissue was collected with a #3 cork borer. For isolates JL-7-2 and ZJ-1-1, inoculated *V. vinifera* cv. Thompson Seedless discs were collected at 24, 48, 72 and 96 hpi. All leaf samples for RNA extraction were snap-frozen in liquid nitrogen and stored at −80 °C until use.

RNA extraction and RNA-Seq analysis

Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma–Aldrich) and DNase-treated according to the manufacturer's instructions. RNA quantity and quality was assessed using a 2100 Bioanalyzer (Agilent Technologies). For isolate CSIRO-L-2, mRNA library construction and Illumina RNA sequencing (single-end, 100 bp reads) were carried out at the Australian Genome Research Facility (Melbourne, Australia). For isolates JL-7-2 and ZJ-1-1, mRNA library construction and Illumina RNA sequencing (paired-end, 2 × 100 bp reads) were carried out at the Novogene Bioinformatics Institute (Beijing, China). Multiplexed barcode sequencing for all samples was performed on a HiSeq 2000 platform.

Read mapping and de novo transcriptome assembly

The raw reads of Illumina sequencing were first checked with FastQC software [20]. Reads with low quality and adaptor contamination (reads with ambiguous bases 'N' and with a base quality less than 15) were removed using the Trimmomatic module in trinityrnaseq_r20140717 package [21]. Reads less than 36 bases after the clipping step were deleted. All processed reads were mapped to the grapevine reference genome (12x) sequence and *V. vinifera* mRNAs from RefSeq database retrieved from the National Centre for Biotechnology Information (http://ncbi.nlm.nih.gov) using TopHatv2.0.8b [22] using default settings. Unmapped reads