EFFECTS OF NITROGEN STARVATION ON EFFECTOR REPERTOIRE OF PROTEINS SECRETED FROM PHYTOPHTHORA INFESTANS (MONT.) DE BARY

PING YU, CHAO DONG, CHUNXIN YAO, XIAOGANG ZHOU* AND YUMEI DING

Institute of Biotechnology and Germplasm Resources, Yunnan Academy of Agricultural Sciences, Ministry of Agriculture, Kunming, Yunnan 650205, China

Keywords: Effector protein, Phytophthora infestans, Nitrogen starvation, Proteome

Abstract

Phytophthora infestans strain NOD-1 was used as experimental material to extract its secreted proteins after nitrogen starvation treatment, and label-free proteomics technology was used to investigate the expression difference of the secreted proteins after various lengths of treatment. By using bioinformatic tools, functional annotation and metabolic pathway analyses were performed for the differentially expressed secreted proteins. According to present results, the expression level of cytoplasmic and extracellular effector proteins increased with the prolongation of treatment time, and also more effector proteins were induced. The expression level of RXLR-like effectors gradually increased under complete culture conditions, and CRN-like effector proteins began to express slightly after 24 days. By contrast, the expression level of CRN-like effector protein increased gradually under nitrogen starvation conditions, and RXLR-like effector protein began to express slightly at 24 days. In addition, except for one small cysteine-rich protein SCR91, all other extracellular effector proteins were highly expressed under complete culture conditions. This study provides a theoretical basis for a more comprehensive understanding of the pathogenesis of P. infestans through functional studies of effector proteins.

Introduction

Phytophthora infestans is an Oomycete that shows different physiological and biochemical characteristics. Its large genetic variation and rapid resistance development make it difficult to prevent and control. So far, potato late blight is still the most devastating disease that threatens the global potato industry. The annual financial loss due to this disease is more than 17 billion US dollars (Haverkort et al. 2008).

During the interaction between pathogens and host plants, the pathogen can secrete some effectors to damage or interfere with the immune response in plants, which promotes the pathogens' infection and propagation (Bozkurt et al. 2012). These effectors are classified, according to their subcellular localization in plants, into two major categories: cytoplasmic effectors and apoplastic effectors. Cytoplasmic effectors mainly include RXLR and CRN effectors (Zhang 2018). Thousands of RXLR effector genes were present in the genomes of Phytophthora, and P. infestans can secrete 563 RXLR effector proteins (Haas et al. 2009, Yin et al. 2017). To date, many studies have revealed that RXLR effectors secreted by P. infestans can regulate the host immune system from multiple aspects, and manipulate the host signaling pathways, thereby showing virulence. In addition to be a virulence protein, the RXLR effector can also function as a non-toxic protein to trigger host HR responses when recognized by the corresponding resistance protein in host plants (Anderson et al. 2015, Du et al. 2015). Similar to RXLR effector, it is known that a few CRN effectors can also manipulate disease resistance pathways of host plants and cell...
death, while most of them can inhibit the inducible resistance generated by plant immune system to promote infection. One *Phytophthora sojae* CRN effector, CRN108, can enter the plant cell nucleus directly and bind to the promoter of the defense-related gene at the heat shock element, preventing the binding of the native transcription factor to the gene and disturbing the gene’s expression. Extracellular effectors mainly include the enzyme inhibitors GIP1 and GIP2, the small cysteine-rich proteins INF1, INF2A, INF2B, PcF and PcF-like SCR74, cysteine, the NEP1-like family of protein NPP1, as well as some glycoproteins, CBEL (cellulose-binding, elicitor, lectin), xylolucan enzymes, and the transglutaminases PEP13 and GP42. They also can stimulate or inhibit the plant immune system.

Studying the secretory proteome of pathogenic fungi under the conditions of induced culture and outside the host has provided useful information for identifying the pathogenic factors. Paper et al. (2007) applied high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) to study the proteome secreted by *Fusarium graminearum*, 49 secreted proteins found only under the conditions of wheat induction, and it is speculated that these secreted proteins play an important pathogenic role during the infection process of *F. graminearum* on in wheat. Wang et al. (2011) used a 2-D method to analyze the secreted proteins of *Magnaporthe oryzae* under nitrogen starvation conditions. Eighty nine differentially expressed secreted proteins were identified, and by using mass spectrometry, they found that these proteins were mainly cell wall hydrolase, esterase and oxidoreductase. The 2-D technique-based study from Yang et al. (2012) investigated the secreted proteome of *F. graminearum* in culture medium with barley and wheat materials as the sole carbon source. Sixty nine differentially expressed secreted proteins were largely cell wall degrading enzymes and starch degrading enzymes. Chu et al. (2015) used LC-MS/MS to analyze the secreted proteome of *Verticillium dahliae* under nitrogen starvation conditions. There are 212 differentially expressed secreted proteins, which mainly include cell wall degrading enzymes, proteases and oxidoreductases.

In this study, a *P. infestans* strain NOD-1 was used as experimental material, and the secreted proteins were extracted after nitrogen nutrient treatment. The label-free proteomics technique was used to compare the expression situations of the secreted proteins after different lengths of treatment. Functional annotation and biological function analysis of differentially expressed secreted proteins were bioinformatically performed, providing a theoretical basis for a more comprehensive understanding of the pathogenic mechanism of *P. infestans* through functional studies of the effector proteins.

**Materials and Methods**

The activation procedure of the *Phytophthora infestans* strain NOD-1 and nitrogen starvation treatment conditions were described previously (Yu et al. 2018). After treating the strain for 8 and 24 day under nitrogen starvation conditions, we used an ammonium sulfate-based extraction method to isolate the secreted proteins. The proteins were subsequently freeze-dried and retained. The protein digestion and mass spectrometry were described previously (Yu et al. 2018).

Gene Ontology (http://geneontology.org/) and KEGG database (https://www.kegg.jp/) were used function and pathway annotation, respectively. The SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/) was also used to analyze the N-terminal signal peptide of the secreted proteins. The subcellular localization of the proteins containing N-terminal signal peptide, using TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/) was predicted. Afterwards, the software TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to analyze the transmembrane structure of the proteins containing N-terminal signal peptide and localized at extracellular domain. All proteins with a predicted transmembrane region were excluded. For all remained proteins, PlIPredictor-Big (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) was employed
to perform GPI anchor location analysis, and proteins containing no anchor sites were also retained. After all the steps, the remaining proteins were considered as classic secreted protein. To obtain non-classical proteins, SecretomeP Server (http://www.cbs.dtu.dk/services/SecretomeP/) through an artificial neural network (NN) algorithm was used. If one protein did not have a predicted N terminal signal peptide and the NN-score was higher than 0.5, it was categorized as non-classical secreted protein.

Results and Discussion

After different time of nitrogen starvation treatment to *Phytophthora infestans*, in total, 5615 peptides and 1188 proteomes were identified by using a label-free proteomics approach. Student’s t test was used to compare the difference between the control group and the treatment group. If the p value is less than 0.05, the protein was considered as a differentially expressed protein.

The expression comparison of the proteins secreted by *P. infestans* grown under nitrogen starvation conditions for 24 and 8 days revealed that 29 proteins were up-regulated, and 4 proteins down-regulated. Fifty two proteins only were present in 24 days nitrogen starvation group, while 8 proteins were in the 8d nitrogen starvation group. Of the 33 significantly differentially expressed proteins, 5 were classical secreted proteins including 2 extracellular effectors, 15 non-classical secreted proteins including 1 extracellular effector and 13 non-secreted proteins including 2 CRN effectors. For the 60 specific expressed proteins, 12 were classical secreted proteins including 2 extracellular effectors, 16 non-classical secreted proteins, and 32 non-secreted proteins including 2 CRN effectors (Table 1). Only 19 of these proteins were involved in the metabolic pathways, and 3 of them were associated with lysosomal metabolism through KEGG analyses.

The expression comparison of the proteins secreted by *P. infestans* after 8 days of nitrogen starvation treatment and complete culture treatment revealed that 4 proteins were up-regulated, and 5 proteins down-regulated. Fourteen proteins only were present in 8 days nitrogen starvation group, while 17 proteins were in the 8d complete culture group. Of the 9 significantly differentially expressed proteins, 1 was classical secreted protein (elicitin), 3 non-classical secreted proteins and 5 non-secreted proteins. For the 31 specific expressed proteins, 5 were classical secreted proteins including 1 extracellular effector and 1 RxLR effector, 7 non-classical secreted proteins, and 19 non-secreted proteins including 1 RxLR effectors (Table 2). Only 15 of these proteins were involved in the metabolic pathways. 6 of them were associated with lysosomal metabolism, and 3 were associated with biosynthesis of amino acids through KEGG analyses.

The expression comparison of the proteins secreted by *P. infestans* after 24 days of nitrogen starvation treatment and complete culture treatment revealed that 33 proteins were up-regulated, and 41 proteins down-regulated. 61 proteins only were present in 24 days nitrogen starvation group, while 24 proteins were present in the 24 days complete culture group. Of the 74 significantly differentially expressed proteins, 22 were classical secreted proteins including 7 extracellular effector and 2 RxLR effector, 20 non-classical secreted proteins including 2 extracellular effector, and 32 non-secreted proteins including 1 extracellular effector, 2 CRN effector and 1 RxLR effector. For the 85 specific expressed proteins, 19 were classical secreted proteins including 3 extracellular effector and 1 RxLR effector, 25 were non-classical secreted proteins including 1 RxLR effector, and 41 non-secreted proteins including 1 CRN effector (Table 3). Only 36 of these proteins were involved in the metabolic pathways. Nine of them were associated with lysosomal metabolism, and 4 were associated with carbon metabolism through KEGG analyses.
Table 1. Mass spectrometric identification of significant differentially secreted proteins of *Phytophthora infestans* after various lengths of nitrogen starvation treatment.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Description</th>
<th>qN24/qN8</th>
<th>P value</th>
<th>Signal P</th>
<th>Target P</th>
<th>TMHMM</th>
<th>PIP</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0RLV7</td>
<td>Transglutaminase elicitor-like protein</td>
<td>2.74</td>
<td>0.0007</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0NV89</td>
<td>Crinkler (CRN) family protein</td>
<td>2.17</td>
<td>0.0009</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.1</td>
</tr>
<tr>
<td>D0NUR9</td>
<td>Crinkler (CRN) family protein</td>
<td>2.05</td>
<td>0.0009</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.1</td>
</tr>
<tr>
<td>D0NUH0</td>
<td>Transglutaminase elicitor, putative</td>
<td>2.99</td>
<td>0.0010</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0MY49</td>
<td>Cellulose binding elicitor lectin (CBEL), putative</td>
<td>2.00</td>
<td>0.0360</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>Q6PQH2</td>
<td>Kazal-like serine protease inhibitor EP11</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>D0N885</td>
<td>Crinkler (CRN) family protein, putative</td>
<td>+/-</td>
<td>-</td>
<td>N</td>
<td>-</td>
<td>1</td>
<td>N</td>
<td>0.1</td>
</tr>
<tr>
<td>D0NUR8</td>
<td>Crinkler (CRN) family protein</td>
<td>+/-</td>
<td>-</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.1</td>
</tr>
<tr>
<td>Q6XDM1</td>
<td>Transglutaminase elicitor M81E</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 2. Mass spectrometric identification of significant differentially secreted proteins of *P. infestans* after 8 days nitrogen starvation treatment and 8 days of complete culture treatment.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Description</th>
<th>qN8/W8</th>
<th>P value</th>
<th>Signal P</th>
<th>Target P</th>
<th>TMHMM</th>
<th>PIP</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q944W0</td>
<td>Elicitin</td>
<td>0.22</td>
<td>0.0127</td>
<td>Y</td>
<td>S</td>
<td>1</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0NGS4</td>
<td>Secreted RXLR effector peptide protein, putative</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0ERS6</td>
<td>Putative RXLR effector PEXRD2_11_8</td>
<td>+/-</td>
<td>-</td>
<td>N</td>
<td>M</td>
<td>-</td>
<td>N</td>
<td>0.7</td>
</tr>
<tr>
<td>Q2M413</td>
<td>Small cysteine-rich protein SCR91</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>1</td>
<td>N</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Table 3. Mass spectrometric identification of significant differentially secreted proteins of *P. infestans* after 24 days nitrogen starvation treatment and 24 days of complete culture treatment.

<table>
<thead>
<tr>
<th>IDs</th>
<th>qN24/W24</th>
<th>P value</th>
<th>Signal P</th>
<th>Target P</th>
<th>TMHMM</th>
<th>PIP</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0NTI4</td>
<td>0.04</td>
<td>0.0000</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.2</td>
</tr>
<tr>
<td>Q9AT04</td>
<td>0.36</td>
<td>0.0001</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0NUH0</td>
<td>0.31</td>
<td>0.0002</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q2N0E4</td>
<td>0.41</td>
<td>0.0002</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>P172</td>
<td>0.9</td>
</tr>
<tr>
<td>Q9S879</td>
<td>0.40</td>
<td>0.0002</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q8d702</td>
<td>0.21</td>
<td>0.0023</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>O42719</td>
<td>0.16</td>
<td>0.0037</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0MY49</td>
<td>0.26</td>
<td>0.0061</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>Q944W0</td>
<td>0.13</td>
<td>0.0076</td>
<td>Y</td>
<td>S</td>
<td>1</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q444V9</td>
<td>0.17</td>
<td>0.0103</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>U3M983</td>
<td>0.43</td>
<td>0.0104</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.2</td>
</tr>
<tr>
<td>D0NQ64</td>
<td>0.26</td>
<td>0.0129</td>
<td>Y</td>
<td>S</td>
<td>1</td>
<td>N</td>
<td>0.6</td>
</tr>
<tr>
<td>Q6PQH2</td>
<td>0.37</td>
<td>0.0426</td>
<td>Y</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>D0NV89</td>
<td>2.94</td>
<td>0.0007</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.1</td>
</tr>
<tr>
<td>Q2M3Z9</td>
<td>2.33</td>
<td>0.0033</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.3</td>
</tr>
<tr>
<td>D0ERS6</td>
<td>+/-</td>
<td>-</td>
<td>N</td>
<td>M</td>
<td>-</td>
<td>N</td>
<td>0.7</td>
</tr>
<tr>
<td>D0MQL8</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.3</td>
</tr>
<tr>
<td>Q2M441</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0NMH3</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0NUG8</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>D0NS85</td>
<td>+/-</td>
<td>-</td>
<td>N</td>
<td>-</td>
<td>1</td>
<td>N</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 4. Mass spectrometric identification of significant differentially secreted proteins of *P. infestans* after 24 and 8 days of complete culture treatment.

<table>
<thead>
<tr>
<th>IDs</th>
<th>Description</th>
<th>W24/W8</th>
<th>P value</th>
<th>SignalP</th>
<th>TargetP</th>
<th>TMHMM</th>
<th>PIP</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0NTI4</td>
<td>Secreted RXLR effector peptide protein, putative</td>
<td>20.52</td>
<td>0.0000</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.2</td>
</tr>
<tr>
<td>D0NUH0</td>
<td>Transglutaminase elicitor, putative</td>
<td>10.15</td>
<td>0.0001</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q9S879</td>
<td>Alpha elicitin, INFESTIN</td>
<td>2.98</td>
<td>0.0001</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q2N0E4</td>
<td>Elicitin-like protein (\text{NL2})</td>
<td>3.18</td>
<td>0.0006</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q9AT04</td>
<td>Necrosis-inducing protein (\text{NPP1})</td>
<td>2.12</td>
<td>0.0008</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q8d702</td>
<td>Small cysteine-rich protein (\text{SCR108})</td>
<td>5.81</td>
<td>0.0015</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0MY49</td>
<td>Cellulose binding elicitor lectin (CBEL), putative</td>
<td>5.44</td>
<td>0.0039</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>O42719</td>
<td>Surface glycoprotein elicitor</td>
<td>3.95</td>
<td>0.0057</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q2N0E6</td>
<td>Elicitin-like protein (\text{NL11B})</td>
<td>2.17</td>
<td>0.0090</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>P,161</td>
<td>0.52</td>
</tr>
<tr>
<td>D0NBQ4</td>
<td>Secreted RXLR effector peptide protein, putative</td>
<td>4.28</td>
<td>0.0111</td>
<td>Y</td>
<td>S</td>
<td>1</td>
<td>N</td>
<td>0.6</td>
</tr>
<tr>
<td>Q944V9</td>
<td>Elicitin-like (\text{INF5})</td>
<td>5.53</td>
<td>0.0115</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q6PQH1</td>
<td>Kazal-like serine protease inhibitor (\text{EPI2})</td>
<td>3.54</td>
<td>0.0181</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>D0RLV7</td>
<td>Transglutaminase elicitor-like protein</td>
<td>11.10</td>
<td>0.0482</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>Q6PQH2</td>
<td>Kazal-like serine protease inhibitor (\text{EPI1})</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>Q6PQG4</td>
<td>Kazal-like serine protease inhibitor (\text{EPI9})</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.6</td>
</tr>
<tr>
<td>Q2M413</td>
<td>Small cysteine-rich protein (\text{SCR91})</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>1</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0MQL8</td>
<td>Secreted RXLR effector peptide protein, putative</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.3</td>
</tr>
<tr>
<td>D0NMH3</td>
<td>Glucanase inhibitor protein, putative</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.9</td>
</tr>
<tr>
<td>D0NUG8</td>
<td>Transglutaminase elicitor, putative</td>
<td>+/-</td>
<td>-</td>
<td>Y</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.8</td>
</tr>
<tr>
<td>D0NUUR8</td>
<td>Crinkler (CRN) family protein</td>
<td>+/-</td>
<td>-</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>N</td>
<td>0.1</td>
</tr>
</tbody>
</table>
The expression comparison of the proteins secreted by *P. infestans* after 24 and 8 days of culture treatment revealed that 43 proteins were up-regulated, and 31 proteins down-regulated. Thirty nine proteins only were present in 24 days complete culture group, while 32 proteins were present in the 24 days complete culture group. Of the 74 significantly differentially expressed proteins, 28 were classical secreted proteins including 7 extracellular effector and 1 RxLR effector, 22 non-classical secreted proteins including 3 extracellular effector, and 24 non-secreted proteins including 1 extracellular effector and 1 RxLR effector. For the 71 specific expressed proteins, 14 were classical secreted proteins including 4 extracellular effector and 1 RxLR effector, 24 non-classical secreted proteins, and 33 non-secreted proteins including 1 extracellular effector and 1 CRN effector (Table 4). Only 37 of these proteins were involved in the metabolic pathways. Nine of them were associated with lysosomal metabolism, and for KEGG terms carbon metabolism, glycolysis/gluconeogenesis and proteasome, they each had 4 proteins enriched through KEGG analyses.

Fig. 1. GO annotation of the differentially expressed proteins after 8 days and 16 hrs of nitrogen starvation treatment.

GO analyses of these differentially expressed proteins in different culture time showed that the major involved biological functions were metabolism, followed by cellular process and single-organism process. The major molecular function terms were catalytic activity and linkage. There was little difference in cell composition, mainly in cell and cell parts.

There are large differences in the secretory proteome of pathogens when they are grown under different culture conditions, or if the length of culture time is different. In this study, the expression level of cytoplasmic and extracellular effector proteins increased with the prolongation of treatment time, and also more effector proteins were induced. The expression level of RXLR-like effectors gradually increased under complete culture conditions, and CRN-like effector proteins began to express slightly after 24 days. By contrast, the expression level of CRN-like
effector protein increased gradually under nitrogen starvation conditions, and RXLR-like effector protein began to express slightly at 24 days. In addition, except for one small cysteine-rich protein SCR91, all other extracellular effector proteins were highly expressed under complete culture conditions.

Fig. 2. GO annotation of the differentially expressed proteins after 8 days of nitrogen starvation treatment and complete culture treatment.

Fig. 3. GO annotation of the differentially expressed proteins after 24 days of nitrogen starvation treatment and complete culture treatment.

The cytoplasmic effector protein RXLR plays an important role in the pathogenesis of pathogenic bacteria. It can not only enhance the pathogenicity of pathogenic bacteria but also be recognized as a non-toxic protein by the resistance protein of the host plant. The RXLR motif of
these effectors bind to the PI3P motif on the host cell membrane and enter the plant cell through a lipid raft mediated endocytosis process (Kale et al. 2010), suppressing the immune system of plants. PexRD2 interacts with MAPKKKe and inhibits its kinase activity, thereby blocking the transmission of MAPK immune signaling pathway and inhibiting plant PTI defense response pathways (King et al. 2014). One typical RXLR effector protein PiAvr3a of *P. infestans* can target and stabilize an E3 ubiquitin ligase CMPG1 in plant cells, thus inhibiting INF-induced necrosis (Bos et al. 2010). In this study, we found the protein expression level was gradually increased in the complete culture condition with the prolongation of treatment time, and there were also more proteins. However, under nitrogen starvation culture conditions, the expression of most proteins only started after 24 days of treatment. These results indicate that complete culture is more conducive to the expression of most effector proteins. Another group of cytoplasmic effectors, CRN. It contains a LXLFLAK motif that is critical to their transport, but the detailed mechanism remains unclear (Schornack et al. 2010). It is known that only a few CRN effector proteins can cause plant cell death, while most of them can inhibit the induced immune resistance in plants. Genomic sequencing results also indicate that there are many CRN effector candidate genes (Haas et al. 2009). In this study, the expression of the effectors from this group increased gradually with the prolongation of treatment time under nitrogen starvation culture conditions, and there were also more CRN effectors under these conditions. By contrast, under the complete culture condition, the expression was slightly increased at 24 days. This difference indicates that nitrogen starvation treatment can disturb the expression levels, and alter the repertoire composition of CRN effectors. Interestingly, we found that all the identified CRN effector proteins contained no predicted signal peptides; yet whether there are new effector protein transport mechanisms remains to be further studied. One previous study showed that the PsIsc1, an effector protein with isoenzyme activity secreted by *P. sojae* did not have a typical signal peptide but could enter plant cells (Liu et al. 2014).

Fig. 4. GO annotation of the differentially expressed proteins after 24 and 8 days complete culture treatment.

Small cysteine-rich proteins can form a disulfide bond, which enhance the stability of the protein in the host extracellular space. With this bond, the protein is not easily degraded and inactivated; thus, it can function as a virulence factor to trigger plant defense responses. However, to date, the detailed mechanism of this process is not yet clear. *P. infestans* secreted SCR74 can
cause cell death on Solanaceae plants; SCR96 and SCR74 play an important role in the colonization and growth of Phytophthora (Liu et al. 2005, Chen et al. 2016). The results of this study indicate that SCR91 was expressed after the strain was grown under nitrogen starvation conditions for 8 days, while its expression only became detectable after 24 days of complete culture treatment. NPP1 is one member of the Nep1-like protein family, and the Nep1 protein is a PAMP molecule widely found in fungi and bacteria (Gijzen and Nurnberger 2006), which plays a role in pathogen’s infection and colonization. The NPP1 from Phytophthora present on the cell wall, and it triggers both PTI defense responses and cell death (Fellbrich et al. 2002, Qutob et al. 2006).

In this study, the NPP1 protein was expressed more highly under complete culture conditions. CBEL is another PNP-specific PAMP whose location is in the cell wall and it is a protein that stimulates necrosis and PTI defense responses (Mateos et al. 1997). CBEL-like domains were identified in 28 proteins of P. infestans, P. sojae, P. ramorum and P. parasitica (Mateos et al. 1997). Mutation of CBEL genes resulted in a thickened cell wall of P. abnormalities (Gaulin et al. 2002) and also caused a decrease in the ability of pathogens to adhere to plant cell walls (Gaulin et al. 2006), indicating this gene’s involvement in the growth and development process of Phytophthora. In this study, the expression level of extracellular effector proteins was increased faster under complete culture conditions and was also higher at 24 days.

Acknowledgements
This study was supported by the Key Project of Yunnan Natural Science Foundation (No.2016FA016) and the Natural Science Foundation of China (No. 31060021).

References
Chen XR, Li YP, Li QY, Xing YP, Liu BB, Tong YH and Xu JY 2016. SCR96, a small cysteine-rich secretory protein of Phytophthora cactorum, can trigger cell death in the Solanaceae and is important for pathogenicity and oxidative stress tolerance. Mol. Plant Pathol. 17(4): 577-587.


Yin J, Gu B, Huang G, Tian Y, Quan J, Lindqvist-Kreuze H and Shan W 2017. Conserved RXLR effector genes of Phytophthora infestans expressed at the early stage of potato infection are suppressive to host defense. Front Plant Sci. 8: 2155.


(Manuscript received on 14 June, 2019; revised on 7 July, 2019)