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Label-free quantitative secretome analysis of *Xanthomonas oryzae* pv. *oryzae* highlights the involvement of a novel cysteine protease in its pathogenicity

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ABSTRACT

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating diseases resulting in a huge loss of the total rice productivity. The initial interaction between rice and *Xoo* takes place in the host apoplast and is mediated primarily by secretion of various proteins from both partners. Yet, such secretory proteins remain to be largely identified and characterized. This study employed a label-free quantitative proteomics approach and identified 404 and 323 *Xoo*-secreted proteins from *in vitro* suspension-cultured cells and *in planta* systems, respectively. Gene Ontology analysis showed their involvement primarily in catalytic, transporter, and ATPase activities. Of a particular interest was a *Xoo* cysteine protease (*Xo*CP), which showed dramatic increase in its protein abundance *in planta* upon *Xoo* interaction with a susceptible rice cultivar. Knock-out mutants of *XoCP* showed reduced pathogenicity on rice, highlighting its potential involvement in *Xoo* virulence. Besides, a parallel analysis of *in planta* rice-secreted proteins resulted in identification of 186 secretory proteins mainly associated with the catalytic, antioxidant, and electron carrier activities. Identified secretory proteins were exploited to shed light on their possible role in the rice-*Xoo* interaction, and that further deepen our understanding of such interaction.
Biological significance: Xanthomonas oryzae pv. *oryzae* (*Xoo*), causative agent of bacterial blight disease, results in a huge loss of the total rice productivity. Using a label-free quantitative proteomics approach, we identified 727 *Xoo*- and 186 rice-secreted proteins. Functional annotation showed *Xoo* secreted proteins were mainly associated with the catalytic, transporter, and ATPase activities while the rice secreted proteins were mainly associated with the catalytic, antioxidant, and electron carrier activities. A novel *Xoo* cysteine protease (*Xo*CP) was identified, showing dramatic increase in its protein abundance *in planta* upon *Xoo* interaction with a susceptible rice cultivar. Knock-out mutants of *XoCP* showed reduced pathogenicity on rice, highlighting its potential involvement in *Xoo* virulence.

1. Introduction

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating diseases of rice, causing up to 80% yield loss in the early stages of the rice growth. Based on the pathogenicity of *Xoo*, the interaction between bacteria and rice can be compatible or incompatible. During compatible interactions, bacteria successfully invade the plants either through the natural openings like hydathodes or through the wounds where it divides in the extracellular spaces of the host including apoplast and xylem. The *Xoo*-rice interaction is mediated largely by the secretory proteins from both the partners (plant and pathogen) in the host apoplast. In general, pathogen-secreted proteins are involved in degradation of plant cell walls, suppression of plant defense responses, and delivery of bacterial DNA and proteins into the host cytoplasm [1], while plant-secreted proteins are mainly involved in the identification of pathogen-derived elicitors and eliciting the defense response [2].

Given the fact that proteins play important roles in determining the fate of the rice-Xoo interaction, a few studies were conducted to identify those proteins [3,4]. A study on plasma membrane proteins isolated from *Xa21*-transgenic rice suspension-cultured cells (SCCs) inoculated with incompatible (PXO99A) and compatible (DY89031) races of *Xoo*

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led to the identification of 20 differentially-modulated proteins, including H-ATPase, protein phosphatase, ascorbate peroxidase, and zinc-finger and Ca($^{2+}$)-binding motif containing (C2 domain protein-like) proteins [3]. Their protein abundance correlated well with their transcript levels upon *Xoo* infection. Another report investigated the xylem sap proteins of an Italian rice cultivar Baldo, infected with *Xoo* and identified 324 *Xoo* proteins in that sap [4]. Furthermore, the knock-out mutant of PruProtein (Xoo1982) showed 34.2% reduction in the virulence in rice leaf [4]. These studies though increased our knowledge on the total and xylem sap proteomes, apoplastic secretory proteins remain yet to be largely investigated, in particular, the *Xoo*-secreted proteins.

Previously, we reported 139 *Xoo*-secreted proteins from *in vitro* SCCs and *in planta* systems using a gel-based proteomics approach [5]. In this extended study, we employed a label-free quantitative proteomics approach for in-depth apoplastic secretome analysis of the rice-*Xoo* interaction using both the *in vitro* SCCs and *in planta* systems.

2. Materials and methods

2.1. Plant material preparation

Rice (*Oryzae sativa*) cultivar Dongjin was used as source material for preparation of apoplastic secretory proteins. Rice cultivars IR24 and IRBB1 were used for pathogenicity assay. Rice seeds were imbibed in sterilized water for two days at 4 °C, transferred to soil for germination for 7 days in growth chamber, followed by transfer of seedling pots to the greenhouse. Mature plants at flowering stage were used for *Xoo* infection.

2.2. Bacterial culture condition and rice leaf infection

Xanthomonas oryzae pv. *oryzae* strain K3 (virulence to rice cultivar Dongjin, IR24, and IRBB1) was grown on PSA agar plate (1% w/v peptone, 1% w/v sucrose, 0.1% w/v glutamic acid, and 1.5% w/v agar) at 28 °C. For *in vitro* culture, *Xoo* strain was inoculated in liquid PSA medium and shaken at 200 rpm in darkness at 28 °C. Cultured *Xoo* cells on PSA medium were collected and washed twice with distilled water. The collected cells were diluted to 1×10^8 cfu/mL (OD = 0.1) with 0.01% tween-20 in water. Rice leaves were cut at the top with sterile scissors, and dipped into the *Xoo* suspension solution twice for 30 s. The infected rice plants were kept in a humidity chamber overnight under darkness at 28 °C, followed by their transfer to normal light (16 h)/dark (8 h) cycles.

2.3. Preparation of secretory proteins

Secretory proteins were prepared from *in vitro* SCCs and *in planta* systems as described previously [5]. *In vitro Xoo*-secreted proteins inside culture medium were collected from 7-day-old SCCs. Briefly, the medium of *Xoo* SCCs was collected by filtering through Whatman filter paper (No. 2), followed by centrifugation at 12,000*g* for 10 min, twice. To the collected medium, Tris-saturated phenol (pH 6.8) was added, mixed gently, and centrifugation at 2500*g* for 15 min. Proteins were precipitated by adding four volumes of methanol containing 0.1 M ammonium acetate to the collected aqueous solution. Precipitated proteins were washed twice with methanol containing 0.1 M ammonium acetate, and then with 80% acetone. The washed proteins were stored in 80% acetone at −20 °C until analysis [6].

In planta secretory proteins were extracted from *Xoo*-infected rice leaves at 10 days post inoculation (dpi). Infected leaves were incubated in the calcium extraction buffer (0.2 M $CaCl_2$, 5 mM Na-Ac, pH 4.3) with gently shaking on ice for 1 h. The extraction buffer was filtered through filter paper and centrifuged at 2500*g* for 15 min at 4 °C. Equal volume of Tris-saturated phenol was added into the extraction buffer and proteins in the phenolic phase were extracted as described above [6]. The purity of prepared secretory proteins was checked as described previously [5,7].

2.4. SDS-PAGE, in-gel digestion, and MudPIT analyses

Equal amount of prepared secretory proteins (30 μg) from three independent biological replicates was loaded and resolved on 12% SDS-PAGE, followed by staining of gels with CBB. For MudPIT analysis, each lane was sliced into five gel fractions (Supplementary Fig. S1). Each gel piece was then subjected to in-gel trypsin digestion as described previously [8,9]. A single phase microcapillary column was constructed with 100 μm i.d. fused silica capillary tubing pulled to a 5 μm i.d. tip by using a CO_2 laser puller (Sutter Instrument Co., CA, USA). The capillary column was packed sequentially with 7 cm of 5 μm i.d. Polaris C18-A (MetaChem Technologies, Torrance, CA, USA) and 3 cm of 5 μm i.d. Partisphere strong cation exchanger (SCX; Whatman, Clifton, NJ, USA), followed by another 3 cm of Polaris C18-A using a home-made high pressure column loader. The columns were equilibrated with 5% ACN/0.1% formic acid (FA) solution. Tryptic peptides were loaded directly onto the capillary column. The buffer solutions used to separate tryptic peptides were: buffer A, 5% (v/v) ACN/0.1% (v/v) FA; buffer B, 80% (v/v) ACN/0.1% (v/v) FA; and buffer C, 500 mM (w/v) ammonium acetate/5% (v/v) ACN/0.1% (v/v) FA. Six steps of SCX-LC/RPLC peptide separation were performed: step 1 (100 min gradient from 0 to 100% buffer B); step 2 (3 min of 100% buffer A); step 3 (2 min of buffer C); step 4 (10 min gradient from 0 to 15% buffer B); step 5 (97 min gradient from 15 to 45% buffer B); and Step 6 (2 min each of 10, 20, 40, 60, and 100% buffer C). As peptides were eluted from the microcapillary column, they were electrosprayed into an LTQ linear ion trap mass spectrometer (Thermo Fisher, CA, USA) with the application of a 2.3 kV spray voltage applied distally to the waste of the HPLCsplit. One full-scan mass spectrum (400–1400 m/z) was followed by nine data-dependent MS/MS spectra at 35% normalized collision energy. The MS/MS spectra were searched as described in the nESI-LC-MS/MS analysis. The maximum number of modifications allowed per peptide was three and the maximum number of modifications per type was five. Common contaminants such as trypsin and human keratins were excluded after searching in the database [10].

2.5. Protein identification and quantification

Acquired spectra were subjected to MaxQuant software (version 1.5.1.2 http://www.maxquant.org/). MaxLFQ procedure, which is integrated into the MaxQuant software employed for label-free data analysis as described before [11], for protein identification and quantification. Protein identification was done by its built-in search engine Andromeda in MaxQuant, against an in-house build combined database of rice and *Xoo* (termed: ricex). The ricex database contains overall 79,763 entries from both rice and *Xoo*. For protein identification, trypsin/p (cleaves after lysine and arginine also if a proline follows) was used for digestion; and maximum two missed cleavages were permitted. Cysteine carbamidomethylation was chosen as fixed modification. N-terminal acetylation and methionine oxidation were chosen as variable modifications. For unmodified peptides, the minimum score and delta score of 0 were used. And for modified peptides, minimum score of 40 and minimum delta score of 6 were employed as threshold for justification. Minimum peptide length was set to seven amino acids. A reverse nonsense version of original database was generated and used to determine FDR. Both peptide and protein FDRs were set to 1% for protein identification as described previously [12]. Minimum one peptide with accepted Andromeda spectra score was used for protein identification [13]. MaxLFQ algorithm was employed for label-free protein quantification, which is integrated into the MaxQuant software [11]. In MaxLFQ, minimum one unique peptide per protein was used for protein quantification. Razor (non-unique) peptides were also used along with unique peptides for quantification. To increase the number of peptides used for

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