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# In-depth insight into in vivo apoplastic secretome of rice-*Magnaporthe oryzae* interaction

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## ABSTRACT

The in vivo apoplastic fluid secretome of rice-blast fungus interaction remains largely uncharacterized. Here, we report a proteomics investigation of in vivo secreted proteins of rice leaves infected with incompatible (KJ401) and compatible (KJ301) races of *Magnaporthe oryzae* (*M. oryzae*) using 2-DGE and MudPIT coupled with MALDI-TOF-MS and/or nESI-LC–MS/MS analyses. Prepared fractions of secretory proteins were essentially free from cytoplasmic contamination. Two-DGE and MudPIT identified 732 secretory proteins, where 291 (40%) and 441 (60%) proteins were derived from rice and *M. oryzae*, respectively. Of these, 39.2% (rice) and 38.9% (*M. oryzae*) of proteins were predicted by SignalP as retaining signal peptides. Among these, rice secreted more proteins related to stress response, ROS and energy metabolism, whereas, *M. oryzae* secreted more proteins involved in metabolism and cell wall hydrolyses. Semi-quantitative RT-PCR revealed their differential expression under compatible/incompatible interactions. In vivo expression of *M. oryzae* glycosyl hydrolase (GH) protein family members using particle bombardment driven transient expression system showed that four GH genes could act as effectors within host apoplast

*Abbreviations:* APF, apoplastic fluid; AFPs, apoplastic fluids proteins; CBB, coomassie brilliant blue; CMC, carboxy methyl celllulose; CWDEs, cell wall degradation enzymes; ECM, extracellular matrix; ECS, extracellular space; GLU2, glucanase 2; G6PDH, glucose 6-phosphate dehydrogenase; GH, glycosyl hydrolase; LSPs, leaderless secretory proteins; MAMP, microbe-associated molecular pattern; MudPIT, multidimensional protein identification technology; 2-DGE, two-dimensional gel electrophoresis; OsPR-10, rice pathogen-related protein 10; PBZ1, probenazole-induced protein 1; PI, post-inoculation; PR, pathogenesis-related; SCCs, suspension-cultured cells; TLP, thaumatin-like protein.

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possibly via interaction with host membrane bound receptor. The established in vivo secretome serves as a valuable resource toward secretome analysis of rice-*M. oryzae* interaction.



## 1. Introduction

The *Magnaporthe oryzae* (*M. oryzae*)-rice (*Oryza sativa* L.) interaction triggers the blast disease, affecting the seed yield and quality of rice that is a staple food for more than half the world's population, especially in South-East Asia [1,2]. To emphasize, the current annual rice production is 651 million tons, out of which more than 90% is grown and consumed in Asia [3]. The rice blast disease alone accounts for an annual yield loss of 10 to 30% of total production despite the cultivation of those cultivars possessing genes imparting resistance against the blast pathogen [1,2]. In recent years, the rice and *M. oryzae* have advanced to become a premier model system for the monocot crop-fungal interaction pathosystem primarily due to complete genome sequence [4–7], resources including mutant populations [8–11], and the depth of numerous studies in both species [1,2,12,14,15]. Numerous targeted studies conducted to date on the rice-*M. oryzae* interaction have refined our knowledge on their interaction mechanisms and responses at molecular, biochemical, and physiological levels [1,2,15]. Our understanding of their interaction biology has benefited tremendously from high-throughput technologies in the genome-geared transcriptomics [16–18], proteomics [12,19], and metabolomics [20] by way of studying dynamics of profiling genes, proteins, and metabolites, respectively, at the whole system level.

During the course of the rice leaf and *M. oryzae* interaction, the extracellular space (ECS) of the rice leaf serves as a front line of defense against the invading *M. oryzae* fungus. Upon interaction, both species secrete a variety of molecular components into the ECS. Secreted molecular components including proteins eventually determine the fate of host as well as pathogen survival. It is likely that the host activates various molecular events like reinforcement of the cell wall, antimicrobial activity by pathogenesis-related (PR) proteins, metabolites, ions, and a large variety of hydrolytic enzymes in the ECS as a concerted defense response against invading *M. oryzae* [13,17]. ECS is also a place where pathogens secrete proteins, such as cell wall hydrolytic enzymes, peptidases, toxins, oxidation/reduction enzymes, and apoplastic effectors, which play a crucial role in infection and pathogenicity [15,21,22]. In addition, host cells recognize extracellular signals through the events in ECS, including microbe-associated molecular patterns (MAMPs) and effectors released from pathogens [21,22].

The high-throughput study of secretory proteins in a desired organism, loosely called secretome [13], has become feasible due to recent advances in plant proteomics technology [23,24]. Plant secretome studies have been performed during its growth and development, and against biotic and abiotic stresses; these studies have been comprehensively reviewed [13]. To date, one study has profiled and cataloged the rice proteins secreted in response to *M. oryzae* and its elicitor using in vitro rice suspension-cultured cells (SCCs) coupled with 2-DGE-based proteomics approach [25]. The study identified 21 differentially expressed protein spots due to *M. oryzae* and/or its elicitor over control [25]. Of nine chitinases found to be secreted, characterization of one chitinase using the in-gel assay revealed strong enzymatic activity. However, it remains to be investigated what is the in vivo secretome of rice leaves upon infection with compatible and incompatible races of *M. oryzae*.

This study aims to identify in vivo proteins secreted into the ECS from both rice and *M. oryzae* at the same time from infected rice leaves using a combination of biochemical and proteomics (gel-based and gel-free) approaches, followed by their correlation assessment with in vivo mRNA expression level using semi-quantitative RT-PCR. Establishment of a new transient assay system for in vivo expression of secreted proteins allowed us to study the function of a subset of *M. oryzae*-secreted proteins, which may act as apoplastic effectors within host apoplast via interaction with host membrane bound receptor. The inventory of the in vivo rice apoplastic proteome in this study will provide important clues in understanding the rice-*M. oryzae* interaction.

## 2. Materials and methods

### 2.1. Preparation of *M. oryzae* fungal pathogen and rice seedling

The conidia (KJ301 and KJ401) were cultured on rice bran agar medium (25 g/L rice bran, 1 g/L sucrose, and 20 g/L agar) and incubated in the dark at 28 °C for 3 days, followed by a further three days incubation under fluorescent light after removal of aerial mycelia as described previously [26]. The japonica rice (*Oryza sativa* L. cv. Jinheung) was used as a primary source of explants. Seedlings were grown for 4 weeks in a green house.

### 2.2. Infection of rice seedlings with *M. oryzae* fungal pathogens

Four-week-old rice seedlings were inoculated with conidial suspension ($1\times10^6$ conidia/mL) of incompatible (KJ401) and compatible (KJ301) races of *M. oryzae* using an air sprayer as described previously [26]. Inoculated plants were kept in a humidity chamber at 28 °C. Leaves were harvested at 72 h post-inoculation (PI) and processed immediately for the isolation of secreted proteins. For transcript profiling, leaf samples were collected at 12, 48, and 72 h PI, frozen in liquid nitrogen, and stored at −70 °C in a deep freezer.

### 2.3. In vitro culture of *M. oryzae* race KJ301

*M. oryzae* was cultured in a complete liquid medium (CM) for 2 days at 28 °C and harvested by vacuum filtration.