Rice Plasma Membrane Proteomics Reveals *Magnaporthe oryzae* Promotes Susceptibility by Sequential Activation of Host Hormone Signaling Pathways

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Plant plasma membrane (PM) plays important roles in immune response. Here, we utilized quantitative mass spectrometry to explore rice PM protein composition and dynamic changes during *Magnaporthe oryzae* infection. We report, thus far, the largest rice PM proteome dataset with 3,906 identified proteins, among which 484 proteins were differentially expressed after *M. oryzae* infection. One third of the identified proteins are predicted to have at least one transmembrane domain. Half of the identified proteins are predicted to have binding functions and over one third of the proteins have enzyme-related functions. In addition, Gene Ontology analyses revealed that abscisic acid (ABA) and cytokinin (CK) signaling were sequentially activated after *M. oryzae* infection in rice. We found that the activation of ABA signaling and the suppression of rice immune response occurred at the early infection stage, while the activation of CK signaling, the upregulation of sugar transporter genes expression, and the nutrient efflux of infected rice cells occurred at later infection stage. Thus, we further propose that *M. oryzae* activates ABA signaling to repress rice immune signaling for initial invasion and redirects nutrient efflux of infected cells for massive growth at the later infection stage.

Plasma membrane (PM) plays a fundamental role in cell activities. It is a biological membrane that separates intercellular cell components from the extracellular environments. Following the identification of PM-located pathogen recognition receptors (PRR) in plants, PM-mediated plant immune responses are becoming a major research focus (Böhm et al. 2014; Monaghan and Zipfel 2012). Many important PRR, such as FLS2, ERF, and CERK1 have been discovered recently that recognize bacterial flagella, EF-Tu, and fungal chitin, respectively (Böhm et al. 2014; Monaghan and Zipfel 2012).

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Rice blast, caused by *Magnaporthe oryzae*, emerges as a major threat to world wide cereal production, causing about 15% annual yield losses (Kim and Lee 2012). *M. oryzae* is a hemibiotrophic pathogen that secretes various virulence effectors into the host cytosol or apoplast to suppress its immune responses during infection (Khang and Valent 2012; Park et al. 2012; Zhang and Xu 2014). Interestingly, during the interaction of rice and *M. oryzae*, plant PM encloses fungal invasive hyphae, forming the plant-derived extra-invasive hyphal membrane (EIHM) (Valent and Khang 2010). In addition, it has been shown that plant PM-derived biotrophic interfacial complex that lies outside of the PM and cell wall of the fungus, preferentially accumulates pathogen-delivered effectors; however, the endoplasmic reticulum-Golgi-dependent secretion pathway accumulates effectors inside the EIHM (Giraldo et al. 2013). In either case, plant-derived PM acts at the forefront of any subcellular organelles in coping with pathogen invasion.

Notably, most of the pathogens live in one or both the host apoplast or xylem, and their proliferation is highly dependent on nutrient availability in these niches (Fatima and Senthil-Kumar 2015). Therefore, the PM-located transporters are frequently targeted or hijacked by pathogens. For example, rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99A delivers type III effector PthXo1 into rice cells during infection. PthXo1 is a transcriptional activator-like effector that directly interacts with the promoter of *OsSWEET11*, a PM-located sugar transporter, and activates its transcription, presumably to induce sugar efflux to apoplast from the pathogen-infected cells (Chen et al. 2010; Yang et al. 2006). However, the nutrient-acquisition mechanisms of other biotrophic or hemibiotrophic pathogens that do not carry PthXo1-like effectors remain largely unknown.

Recently, advancements of quantitative mass spectrometry allow us to globally and systemically profile the protein expression at subcellular levels. In this study, we employed the isobaric tags for relative and absolute quantitation (iTRAQ) peptide labeling approach and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for quantitative analyses of the PM proteome of *M. oryzae*-infected rice leaves. For the first time, close to 4,000 proteins were identified as PM-located or -associated proteins, providing a general view of plant PM protein composition. Most importantly, such datasets allow us to gain deeper insights than ever before on PM protein dynamics and their roles during pathogen infection.

RESULTS

Differential analyses of the PM proteins of rice leaves upon *M. oryzae* infection and their functional annotations.

Because of the importance of PM in plant immunity, we employed quantitative proteomics to dissect rice PM protein constitution and dynamic changes when the plants were subjected to *M. oryzae* infection. To find the critical time points for samplings, we first examined fungus development in rice cells. At 24 h postinoculation (hpi), fungal hyphae exclusively grew in first-infected cells, while at 36 hpi, they had invaded the neighboring cells (Fig. 1A). At 48 hpi, the fungi had crossed the second-infected cells and invaded the third cells. Importantly, 3, 3'-diaminobenzidine (DAB) staining indicated H_2O_2 was just-produced at 36 hpi but extensively produced at

48 hpi. Therefore, we decided to sample the PM proteome at 24 and 48 hpi.

PM proteins were extracted from the rice leaves of both *M. oryzae* and mock-inoculated seedlings, using aqueous two-phase partitioning. The resulting PM purity was examined by immunoblotting against specific markers (Fig. 1B). Total proteins in the samples were enzymatically digested and were labeled with iTRAQ reagents prior to protein identification and quantification using LC-MS/MS. In total, we identified 3,906 rice PM proteins, the largest proteomic dataset of its kind (Fig. 1C; Supplementary Table S1). Of all protein identifications, we found 3,052 proteins with annotated biological functions and 854 assignments without known functions (Fig. 1E). Transmembrane hidden Markov model (TMHMM) software was then

Fig. 1. Experimental design and characterization of rice plasma membrane (PM) proteins. **A**, Fungal hyphae growth in rice sheath cells. Rice sheath cells were inoculated with conidial suspensions (1×10^5 conidia per milliliter in 0.2% Tween-20) and were stained with 3, 3'-diaminobenzidine. Images were taken by a light microscope (Olympus BX51) at indicated times. **B**, Immunoblot assay of the PM enrichment. The enriched PM were examined by immunoblotting. Crude leaf extract, microsomes, and enriched PM were immunoblotted with indicated antibodies. The respective antibodies within the parentheses indicate the subcellular localization. **C**, Schematic diagram of the proteomics workflow. The pathogen- and mock-treated rice leaves were used to purify PM proteins. The enriched PM proteins were digested with trypsin and then labeled with isobaric tags for relative and absolute quantitation (iTRAQ) reagents. The labeled peptides were subjected to fractionation and liquid chromatography-tandem mass spectrometry analyses. **D**, Prediction of transmembrane domains. The differentially expressed proteins were analyzed for the numbers of transmembrane domains by TMHMM software (TMHMM 2.0c version) on the Linux platform. **E**, Molecular function analysis of PM proteome. In total, 3,906 PM proteins were subjected to Gene Ontology analysis for biological functions. The database was obtained from the Rice Genome Annotation Project.

used for prediction of transmembrane helices of all identified proteins *in silico*. The results show that about 32% (1,240 of 3,906) of the identified proteins contain one or more transmembrane domains (Fig. 1D). Furthermore, more than 540 proteins have one transmembrane domain, including 170 identified receptor-like kinases (RLKs) in this study. Most single transmembrane domain-containing proteins have catalytic or kinase activities (34.16%), and the rest have binding functions (43.37%). Multiple transmembrane domain-containing proteins tend to have transporter activity. More than 170 proteins contain nine to 12 transmembrane domains, in which 66.3% of them have transporter activity. In addition, the proteins without a transmembrane domain are likely to be peripheral proteins. Indeed, 68.2% of those proteins have binding functions, such as protein- or lipid-binding functions.

For functional classification, 1,824 proteins were classified as having a binding function, which are predicted to bind proteins or nucleotides. Of all 3,906 proteins, the enzyme-related proteins account for 36.8% (1,436 proteins), of which 683 proteins are predicted with kinase activity, 501 proteins with hydrolase activity, 234 proteins with transferase activity, and only 50 proteins with enzyme regulator activity, indicating protein kinases and hydrolases are the major enzymes on rice PM. In addition, 683 proteins are predicted with catalytic activity and 364 proteins are predicted with transporter activity. However, the proteins that are predicted to be either structural molecules or signal transducers and receptors only account for 4.5 and 1.54%, respectively (Fig. 1E). These results indicate that plant PM proteins are primarily made up of binding and enzymatic proteins and that the transmembrane proteins are made up of transporters, catalytic proteins, or kinases.

Homology-based annotation and enrichment analysis of differentially expressed proteins (DEPs).

Of the 3,906 proteins, 484 DEPs were identified for further analysis. In the 24-hpi samples, 92 proteins were found to be upregulated and 65 proteins were found to be downregulated, while in the 48-hpi samples, 188 proteins were found to be upregulated and 168 were found to be downregulated (Fig. 2A).

To further clarify the functions of the DEPs at different time points (24 and 48 hpi), a homology-based analysis of all up- and downregulated proteins were performed by Blast2Go software. Gene Ontology (GO) analysis shows DEPs cover a wide range of molecular functions, such as protein binding (24 hpi, 51.59%; 48 hpi, 37.36%), transmembrane transporter activity (24 hpi, 8.28%; 48 hpi, 12.08%), and enzyme-related functions. The enzyme-related proteins are further categorized as having oxidoreductase activity (24 hpi, 14.01%; 48 hpi, 14.89%), kinase activity (24 hpi, 14.65%; 48 hpi, 12.08%), phosphotransferase activity (24 hpi, 12.74%; 48 hpi, 10.67%), and hydrolase activity (24 hpi, 14.01%; 48 hpi, 15.71%), respectively (Fig. 2B). The most abundant biological processes were classified to be involved in innate immune response (24 hpi, 18.47%; 48 hpi, 14.61%), signal transduction (24 hpi, 27.39%; 48 hpi, 18.54%), response to biotic stimulus (24 hpi, 31.85%; 48 hpi, 28.65%), response to hormone stimulus (24 hpi, 28.66%; 48 hpi, 17.70%), transport (24 hpi, 36.94%; 48 hpi, 34.27%), response to abiotic stimulus (24 hpi, 37.58%; 48 hpi, 30.34%), and RLKs (24 hpi, 15%; 48 hpi, 23.8%) (Fig. 2B).

Immune receptors and signaling proteins often localize at the PM as integral or peripheral proteins (Böhm et al. 2014; Monaghan and Zipfel 2012). In our study, we found many PM DEPs were assigned to immune system process and were up-regulated in 48-hpi samples (Fig. 2C; Supplementary Fig. S2). For example, NADPH oxidase plays a pivotal role in generating reactive oxygen species (ROS) during pathogen infection (Wong et al. 2007). *Osrboh8* (LOC_Os11g33120), which

encodes a rice NADPH oxidase, was up-regulated in 48-hpi samples. In addition, we detected an E3 ubiquitin ligase OsPUB15 (LOC_Os08g01900) that alleviates ROS stress and reduces cell death (Park et al. 2012) was up-regulated in 48-hpi samples. Furthermore, two nucleotide binding-leucine-rich repeat (NB-LRR) disease resistance proteins, LOC_Os01g36640 and LOC_Os11g11960 (rice homologs of *Arabidopsis* PM-associated protein RPM1) were up-regulated in 48-hpi samples. However, many fewer proteins were up-regulated in 24-hpi samples (Fig. 2C). The most significantly upregulated proteins are vesicle trafficking-related proteins, such as LOC_Os08g41340, LOC_Os06g45380, and LOC_Os06g07200 in 24-hpi samples, while the downregulated proteins are RNA recognition motif-containing proteins, such as NADPH quinone oxidoreductase and decarboxylase. These data indicated that a distinct set of proteins was induced in 48- versus 24-hpi samples, implying that the plant immune system was differentially regulated at 24 and 48 hpi.

GO analysis revealed that ABA and CK signaling pathways were sequentially activated.

To understand the biological significance underlying differentially regulated proteins, over-represented processes were determined relative to the background set of all identified proteins. Results of significantly enriched GO biological process terms in 24- and 48-hpi samples are presented in Figure 3A and B, respectively. Several highly enriched processes in 24-hpi samples appear in the 48-hpi samples as well, such as monosaccharide metabolic, lipid biosynthetic, and sulfur metabolic processes, indicating these processes are constitutively activated, at least, before 48 hpi. However, some biological processes, such as transmembrane transport and disaccharide metabolic and isoprenoid biosynthetic processes, were only detectable in 48-hpi samples.

Interestingly, two distinct hormone-related processes among others were highly enriched in 24- and 48-hpi samples, respectively (Fig. 3A and B). The DEPs that are responsive to ABA and CK stimuli are reported in Figure 3C. A heat map showed that the ABA-responsive proteins were up-regulated at 24 hpi but decreased at 48 hpi; whereas, the CK-responsive proteins were extensively induced at 48 hpi. Although ABA-responsive genes could be detected until 48 hpi, Figure 3C (left panel) and Figure 4A clearly show that ABA signaling is primarily activated at 24 hpi or even earlier. Most importantly, ABA contents are higher at 24 than at 48 hpi (Fig. 4A). Therefore, ABA response at 48 hpi is likely approaching the end of signaling activation. Similar to ABA, CK signaling is mainly activated at 48 hpi, although few CK-responsive genes were up-regulated at 24 hpi (Fig. 3C).

Although almost equal amount of JA signaling-related proteins (27 proteins) were differentially regulated, they were not significantly enriched by GO analysis, probably because jasmonic acid (JA)-responsive proteins were up-regulated before the 24-h time point (Yang et al. 2016). Nevertheless, the above data suggest that rice ABA and CK signaling are sequentially regulated after *M. oryzae* invasion.

Activation of ABA signaling compromised disease resistance to *M. oryzae*.

The above-mentioned GO analyses suggest that ABA signaling was activated in rice at 24 hpi (Fig. 3A). To verify whether ABA signaling was indeed activated, we examined the transcription of ABA-responsive gene *salt* by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). As shown in Figure 4A, *salt* transcription was induced rapidly after pathogen treatment (at 6 hpi) and then decreased gradually. Next, we measured the ABA contents in both pathogen and

mock-treated rice leaves. We found *M. oryzae* infection resulted in ABA accumulation at 24 hpi (Fig. 4B).

Spence et al. (2015) have shown that *M. oryzae* produces and secretes ABA to host cells to enhance susceptibility. Therefore, to define the origin of ABA, we examined transcription of the ABA biosynthesis genes *OsNCED1*, *OsNCED2*, *OsNCED3*, *OsNCED4*, and *OsZEP1*, by qRT-PCR. We found the transcription of all tested genes was significantly induced by pathogen treatment (Fig. 4C), demonstrating ABA is de novo-synthesized after *M. oryzae* invasion. Next, we examined expression of the putative ABA transporter gene. Interestingly, we found that these genes were also induced upon *M. oryzae* infection (Supplementary Fig. S3). Thus, we speculate that the rice ABA transporter may be involved in ABA uptake from the pathogen.

ABA has been demonstrated to antagonize salicylic acid (SA) signaling to suppress immune response in rice (Jiang et al. 2010). Indeed, ABA treatments suppressed the SA-responsive gene *OsNPR1*, *OsPRIa*, and *OsPRIb* transcription in Zhong-10-8-14-treated rice leaves (Fig. 4D). Interestingly, ethylene (ET) and JA signaling was repressed as well, as transcription of the ET-responsive gene *OsERF073* and the JA-responsive gene *OsJAR1* declined. To determine whether ABA signaling was involved, we inoculated the ABA-insensitive mutant *Osabi3* with *M. oryzae*. ABI3, one of the B3 transcriptional regulators, regulates ABA-dependent gene expression. The result showed that the *Osabi3* mutant was resistant to *M. oryzae* infection. In contrast, exogenous application of ABA severely impaired disease resistance in wild-type (WT) plants (Fig. 4E and F).

Fig. 2. Gene Ontology biological process of differentially expressed proteins (DEPs). **A**, DEPs at different time points. The pathogen- and mock-treated rice leaves were used for plasma membrane protein enrichment. The detected proteins compared with their respective datasets with the threshold of log2-fold change ≥ 0.5 or ≤ -0.5 were considered as DEPs. The number of DEPs are displayed by 24 and 48 hpi, respectively. **B**, Classification of the DEPs into molecular functions and biological processes. The homology-based annotation was performed by Blast2Go software to analyze the DEPs. The darker shaded slices represent upregulated proteins and the lighter shaded slices represent downregulated proteins. **C**, Heat map representing the differentially regulated immune proteins. The DEPs were subjected to homology-based annotation by Blast2GO against the Rice Genome Annotation Project database.

These results suggest *M. oryzae* infection activates ABA signaling and ABA acts as a negative regulator for disease resistance in rice.

To learn how ABA affects plant immunity, we blasted all the DEPs against microarray data from the publicly available Rice DB database for abiotic stress stimuli, which presumably is ABA-dependent. More than half of the DEPs could be retrieved from the database, suggesting ABA-mediated immune suppression is probably attributable to the activation of abiotic stress signaling (Supplementary Fig. S4). Furthermore, we performed transcriptome analyses for *M. oryzae*-infected rice leaves. The result showed that the transcriptions of ABA and abiotic stress-related genes, indeed, were up-regulated in WT plants after pathogen treatment (Fig. 4G). Of all the differentially expressed genes, the abiotic stress-related genes account for about 8.9%, indicating *M. oryzae* inoculation leads to the reprogramming of abiotic stress-responsive genes at the genome level (138 of 1,535) (Fig. 4G; Supplementary Fig. S5; Supplementary Table S2).

Activation of CK signaling during *M. oryzae* infection.

Because the proteome data indicated the CK signaling pathway was activated at 48 hpi, we explored the roles of CK during *M. oryzae* infection. First, we examined whether a *M. oryzae* strain, Zhong-10-8-14, could affect CK content in

rice leaves. Similar to a previous study (Jiang et al. 2013), we found the fungal strain Zhong-10-8-14 could also efficiently induce CK accumulation (Fig. 5A). Notably, the CK levels of inoculated leaves are higher at 48 than at 24 hpi, implying CK may function at the later infection stage. Next, we examined the transcription of those genes that are involved in plant CK biosynthesis. The results showed that CK biosynthesis gene transcription was not affected by *M. oryzae* infection (Supplementary Fig. S6), suggesting that the elevated level of CK in the infected rice leaves was not due to de novo biosynthesis.

Then, we investigated whether elevated CK levels activate downstream gene expression after pathogen inoculation. We chose *OsRR* family genes as CK signaling-responsive genes (Jain et al. 2006). As shown in Figure 5B, transcription of the *OsRR* gene was highly induced, indicating CK signaling was activated. These results indicated that *M. oryzae* infection induces CK accumulation as well as CK signaling activation in rice. This result is consistent with the proteomic data (Fig. 3B). However, the origin of CK requires further investigation in the *M. oryzae*-infected plants.

Activation of the CK signaling promotes accumulation of nutrients in rice apoplast.

GO analyses of biological processes showed that, along with the activation of CK signaling, the carbohydrate and lipid biosynthesis

Fig. 3. Gene ontology (GO) analysis revealed sequential activation of abscisic acid (ABA) and cytokinin (CK) signaling pathways. **A**, Over-represented GO biological processes in 24-h postinoculation (hpi) proteome dataset. A total of 157 differentially expressed proteins (DEPs) were subjected to GO analysis. Beijing Genomics Institute's WEGO tool was used to detect GO biological process over-representation in the 24- and 48-hpi DEP groups, respectively. The bar chart shows fold enrichment of GO biological process terms in the upregulated proteins relative to background set of all identified proteins. The significant representative term in each cluster is reported. The terms are ordered from least significant (top) to most significant (bottom). ***, $P < 0.01$; **, $P < 0.05$; *, $P < 0.1$. **B**, Over-represented GO biological processes in 48-hpi proteome dataset. A total of 356 DEPs were subjected to GO analysis. Data analysis is the same as in A. **C**, Heat map demonstrating the over-representing DEPs in ABA and CK signaling. DEPs are listed for ABA signaling (left) and CK signaling (right). The DEPs were subjected to homology-based annotation by Blast2GO against the Rice Genome Annotation Project database. Black represents protein undetected in this sample.

were significantly up-regulated (Fig. 3B). Elevation of metabolite biosynthesis suggests nutrient competition becomes the major event for fungi-host interactions at 48 hpi (Fig. 3B). Notably, at this stage, the most obvious sign is massive growth of the fungi. This reminds us that fungi must deploy some strategy to dictate nutrient mobilization from host cytosol to apoplast. Therefore, we performed metabolite profiling analysis for rice apoplast substances, by gas chromatography-mass spectrometry (GC-MS).

Prior to the analysis, we checked the purity of apoplast washing fluid (AWF). The commercially available glucose 6-phosphate dehydrogenase (G6PDH) and malate dehydrogenase (MDH) assay kits were used for purity assessment. The results showed that the cytosol contaminants were negligible for this assay (Supplementary Fig. S7). As shown in Table 1, GC-MS detected about 20 compounds with the signal/noise ratio greater than 5 (Table 1). Majority of the detected compounds were increased after pathogen treatment. For instance, the level of D-glucose increased by almost an order of magnitude and the levels of L-proline, sucrose, fructose, and mannose are approximately two-fold greater than mock treatment. These results clearly demonstrate *M. oryzae* infection results in the efflux of nutrients from host cytosol to apoplast.

Next, we questioned how the fungi co-opt host processes to promote the efflux of the nutrients from cytosol. In addition to metabolite biosynthesis process, the GO analyses also reveal that the transmembrane transport systems were up-regulated at 48 hpi, including ATPase, sugar transporters, and ABC transporters (Figs. 3B and 6A). It is known that PM ATPases and ABC transporters are involved in substance transportation. Notably, we found the upregulation of sugar transporters in the samples of 48 hpi. Previous studies showed that CK has been implicated in the mobilization of nutrients toward the infection site and induces sink effects (Jiang et al. 2013; Walters et al. 2008). Therefore, we examined whether CK treatment could induce the nutrient efflux in rice. We determined the levels of the apoplast sugars after CK treatment by using LC-MS/MS in the multiple reaction monitoring (MRM) mode. As shown in Figure 6B and C, CK treatment was able to induce glucose and fructose accumulation in rice apoplast.

It is known that plant invertase hydrolyzes sucrose to fructose and glucose. To ascertain whether the invertase contributes to the accumulation of fructose and glucose in rice apoplast after CK treatment, we measured the transcription of rice invertase gene. We found that invertase transcription was induced by both pathogen and CK treatments (Supplementary Fig. S8C).

Fig. 4. Activation of abscisic acid (ABA) signaling compromised disease resistance to *Magnaporthe oryzae*. **A**, ABA-responsive gene *SalT* expression over time after *M. oryzae* infection. Conidial suspensions (1×10^5 conidia per milliliter in 0.2% Tween-20) were sprayed onto the leaf surface of two-week-old seedlings. The ABA-responsive gene *SalT* was chosen for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis at indicated times. The experiments were repeated at least three times. **B**, ABA contents in pathogen-infected rice leaves. The treatment of rice seedlings is same as in **A**. **C**, The ABA biosynthesis gene expression in response to *M. oryzae* invasion. The ABA biosynthesis genes *OsNCED1*, *OsNCED2*, *OsNCED3*, *OsNCED4*, and *OsZEP1* transcription levels were determined by qRT-PCR at 24 h postinoculation (hpi). Pathogen treatments are the same as in **A**. **D**, The disease-responsive gene expression in response to ABA treatment. The pathogenesis-related gene expressions were examined by qRT-PCR after exogenous 100 μ M ABA treatments for 6 h. The experiments were repeated at least three times. **E**, ABA signaling regulates disease resistance of rice seedlings. Pathogen inoculation is the same as in **A**. ABA (100 μ M) was sprayed onto rice leaves as pretreatment. The pretreated rice leaves were inoculated 24 h later with *M. oryzae* conidia. The image was taken at 5 days postinoculation. Bar = 1 cm. **F**, Number of disease lesion per leaf shown in **E**. * and ** represent significant difference at $P < 0.05$ and $P < 0.01$ levels, respectively. The experiments were repeated at least three times. **G**, Venn diagram showing differential expression of ABA and abiotic stress-related genes in *M. oryzae*-infected rice leaves. Rice plants (WT) were pathogen- and mock-treated as described in **A**. At 24 hpi, the plants were sampled for transcriptome analysis.

Meanwhile, we examined whether CK could regulate transporter gene expression. After CK treatments, the transcript levels of sugar transporter genes as well as ATPase and ABC transporter genes were significantly higher (Fig. 6D), indicating CK-mediated transporter gene expression is probably responsible for the sugar efflux. Because some of these genes are annotated as monosaccharide transporters, we speculate that PM-located sugar transporters, at least in part, facilitate fructose and glucose accumulation in rice apoplast during pathogen infection; however, the elevated invertase transcripts suggest a potential role of invertase as well. Nevertheless, these data suggest CK is involved in the efflux of cytosol nutrients to apoplast during *M. oryzae* infection.

DISCUSSION

Rice PM proteome and their immune responses during *M. oryzae* infection.

Owing to the abundant chloroplast proteins, purification and enrichment of plant PM proteins are very difficult. In this study, we took advantage of iTRAQ labeling and the latest Orbitrap Fusion technology and observed more than 3,900 proteins in highly purified rice PM. Of the identified proteins, 32% are predicted to be PM proteins and 36.8% are enzymes. In particular,

683 proteins are kinases and 501 proteins are hydrolases, followed by transferases (234) or enzyme regulators (50). Because of the importance of transporters, it is not surprising that 11.9% (364 of 3,906) of identified proteins have transporter activities. Proteins with catalytic activity are categorized as enzymes, which account for 17.5% (683 of 3,906). However, the most abundant proteins are annotated to have binding functions. These proteins are involved in protein, nucleotide acid, or other substance binding, accounting for almost 50% of the identified proteins (Fig. 1E). Thus, our comprehensive dataset provides a general view of plant PM protein composition.

GO biological process reveals plant immune systems are differentially regulated between 24 and 48 hpi. For example, the upregulation of phospholipase proteins is exclusively found at 24 hpi. Plant phospholipases are involved in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) by producing phosphatidic acid, which plays various roles in ROS production, mitogen-activated protein kinase signaling, ET signaling, and direct targeting of defense-related gene expression and are linked to the resistance response to *M. oryzae* in rice (Canonne et al. 2011; Song and Goodman 2002). Similarly, many vesicle trafficking-related proteins were identified to be upregulated at 24 hpi. Although the information of vesicle

Fig. 5. Pathogen treatment triggers cytokinin (CK) accumulation in rice leaves. **A**, Transzeatin levels after pathogen treatment. Conidial suspensions (1×10^5 conidia per milliliter in 0.2% Tween-20) were sprayed onto the leaf surface of two-week-old seedlings. Transzeatin contents of rice leaves were measured by liquid chromatography-tandem mass spectrometry. ** represents the significant level at $P < 0.01$. **B**, CK-responsive gene expression after *Magnaporthe oryzae* treatment. Pathogen treatments are the same as in A. The expression of the *OsRR* gene family, which are defined as CK-responsive genes, were examined by quantitative reverse transcription-polymerase chain reaction analysis at 48 h postinoculation.

Table 1. Gas chromatography-mass spectrometry (GC-MS) analysis of apoplast metabolites in pathogen-infected rice leaves^a

No.	Compound name	Elution time (min)	Similarity	Fold change
1	Propanoic acid	5.36	95	1.95 ± 0.11**
2	N-methyl-phenylethanolamine	5.90	71	0.97 ± 0.01
3	Ethanedioic acid	6.06	80	0.78 ± 0.01***
4	4-hexen-2-one	6.88	69	0.89 ± 0.22
5	Silanol	7.29	91	1.01 ± 0.28
6	Silanolamine	8.80	87	1.34 ± 0.20
7	Butanoic acid	9.28	68	1.17 ± 0.09
8	Cadaverine	9.48	80	0.86 ± 0.14
9	L-proline	9.60	94	2.47 ± 0.18**
10	D-fructose	12.36	94	2.17 ± 0.26**
11	L-sorbose	12.42	90	1.25 ± 0.03**
12	D-glucose	12.54	81	8.72 ± 1.48**
13	Mannose	12.68	81	1.89 ± 0.64*
14	Hexadecanoic acid	13.31	95	0.45 ± 0.22*
15	Myo-inositol	13.84	88	0.96 ± 0.56
16	Octadecanoic acid	14.54	94	1.36 ± 0.86
17	Sucrose	16.98	90	2.80 ± 0.35*

^a The rice apoplast washing fluid compounds were determined by GC-MS. The compounds with signal/noise ratios >5 are reported. *, **, and *** represent $P < 0.1$, $P < 0.05$, and $P < 0.01$, respectively. Fold change was presented against mock control as means ± standard deviation ($n = 3$).

trafficking-related proteins is limited in rice, they have been suggested to participate in PAMP receptor recycling of *Arabidopsis* (Wang et al. 2016).

Interestingly, we detected two putative NB-LRR disease-resistance proteins and several RLKs in upregulated DEPs at 48 hpi but not at 24 hpi, suggesting that there were effector recognition events occurring at 48 hpi. Many RLKs are also significantly upregulated at 48 hpi. RLKs play important roles in recognition of PAMPs, such as LRR-RLK FLAGELLIN SENSITIVE2 (FLS2) and chitin elicitor receptor kinase 1 (CERK1), which mediate perceptions of bacterial flagellin and fungal chitin and peptidoglycan, respectively (Antolín-Llovera et al. 2014). Because these RLKs have not yet been characterized, uncovering their functions will help to understand rice innate immunity. Further, almost all the downregulated proteins are redox- or cell death-related, such as the putative mitochondria carrier protein, mitochondria prohibitin complex protein, NADP reductase

and peroxisomal biogenesis factor, implying the cells were dying at 48 hpi, although we did not see visible cell death. Because we observed many PTI-responsive proteins at 24 hpi and cell death-related proteins at 48 hpi, we proposed that PTI primarily is activated within 24 hpi and effector-triggered immunity is activated at around 48 hpi.

ABA in blast resistance.

Cumulating evidences indicate ABA is involved in plant immune response (de Torres Zabala et al. 2009; Jiang et al. 2010). In rice, exogenous ABA significantly promotes the blast disease severity by suppressing *WRKY45* and *OsNPR1* transcriptions (Jiang et al. 2010; Ueno et al. 2015). Furthermore, *M. oryzae*-derived ABA is presumably to activate rice ABA signaling during infection (Jiang et al. 2010; Spence et al. 2015). Interestingly, our proteome data suggest that ABA signaling is preferably activated at early infection stage (around 24 hpi or

Fig. 6. Effects of cytokinin (CK) on apoplast sugar accumulation. **A**, Heat map demonstrating upregulated transporter proteins. The differentially expressed proteins of the 48-h postinoculation proteome dataset were annotated against the Rice Genome Annotation Project database and were selected for clusters that are characterized as transporters and transporter-related proteins. **B**, CK facilitates sugar accumulation in rice apoplast. Rice leaves were incubated with 200 μM CK for 6 h before apoplast sugar determination. The rice apoplast sugars were examined using liquid chromatography-tandem mass spectrometry in the multiple reaction monitoring mode with the following transitions: *m/z* 179 > 89, fructose; 179 > 89, glucose. Image shows the ion intensities of fructose and glucose. **C**, The absolute contents of sugars are drafted as charted in B. Standard deviation represents triplicates (*n* = 3). ** represents *P* < 0.05. **D**, CK regulates the expression of the putative sugar transporter genes. Two putative monosaccharide transporter genes (LOC_Os03g11900 and LOC_Os07g37320) and two putative transporter family genes (LOC_Os07g39350 and LOC_Os09g24924) were selected for quantitative reverse transcription-polymerase chain reaction analysis. CK treatments are the same as in B.

earlier). At this stage, fungal growth is exclusively limited in the first-infected cells (Fig. 1A). Because ABA negatively modulates disease resistance, we speculate that *M. oryzae* infection provokes host ABA signaling at the early stage to suppress plant immunity. Recently, Ueno et al. (2015) reported that abiotic stresses could antagonize the rice blast defense through deactivation of SA signaling. In the presence of exogenous ABA or under abiotic stresses, OsWRKY45-mediated disease resistance was impaired (Ueno et al. 2015). This study indicates that the activation of abiotic stress signaling impairs plant immune response.

ABA has long been known as an abiotic stress hormone and is induced by diverse abiotic stresses, such as low temperature, salt, and drought stresses. The GO analysis indicates that the abiotic stress-related proteins are remarkably accumulated (Fig. 3A). The comparison with the published transcriptome data shows that more than 50% of DEPs are involved in all abiotic stresses. Our transcriptome data acquired by pathogen-infected materials further reinforce this conclusion (Fig. 4G). Therefore, ABA-mediated immune suppression is probably due to the activation of abiotic stress signaling at 24 hpi.

CK in plant immunity.

CK is recently recognized as an important phytohormone that is linked to plant immunity (De Vleeschauwer et al. 2014). Elevated CK levels have been found to either enhance or suppress plant immunity, depending on the specific pathogens or the infection strategies used by pathogens (Argueso et al. 2012; Choi et al. 2010; Naseem and Dandekar 2012; Reusche et al. 2013). Recently, CK has been reported to coordinate with SA to defend against *M. oryzae* infection; however, CK treatments induce neither SA accumulation nor disease resistance (Jiang et al. 2013), implying CK has additional roles.

Genome annotation of *M. oryzae* does not show there are obvious PthXo1-like effectors (Dean et al. 2005). Apparently, *M. oryzae* might employ other strategies rather than using effectors to activate “sweet” genes. The proteomic data along with apoplast metabolite profiling reveal that *M. oryzae* co-opts host transportation machinery to acquire nutrients in apoplast, partly through CK signaling activation (Fig. 6; Table 1). CK treatment results in the upregulation of sugar transporter-encoded genes (Fig. 6D), suggesting PM-located transporters mediate the nutrient efflux. Notably, these transporters accumulate at 48 hpi, but just a few are detectable at 24 hpi, indicating time-dependent accumulation. Our data suggest that CK plays a

negligible role in immune responses; rather, it is an “old fashioned” substance that probably only affects plant growth, such as nutrient distribution. Because, at the later infection stage, regardless of the origin of CK, the infection sites accumulate higher levels of CK and nutrients, exogenous applied CK would not change the development and pathogenesis of rice blast. Our results could explain the pending question about why exogenous CK could not affect plant disease resistance (Jiang et al. 2013). Therefore, we describe a mechanism by which *M. oryzae* infection activates CK signaling and, subsequently, induces transporter gene expression, leading to the export of assimilates from the cytosol to the apoplast in rice.

Conclusion.

In summary, we provide a general view of rice PM protein composition and its dynamic changes after *M. oryzae* infection. More importantly, we found *M. oryzae* sequentially activated host hormone pathways to benefit its growth. We showed that *M. oryzae* activates ABA signaling to repress rice immune signaling for initial invasion and to redirect nutrient efflux of infected cells for massive growth at the later infection stage (Fig. 7). How the pathogen programs the infection strategies needs further investigation. Future efforts should be made to manipulate ABA and CK signaling pathways in rice blast disease tolerance breeding.

MATERIALS AND METHODS

Plant and fungi growth conditions.

The rice (*Oryza sativa*) variety Nipponbare (WT) and ABA-insensitive mutant (*Osabi3*) were grown in a controlled environmental chamber at 28°C with a 16-h-light and 8-h-dark photoperiod and 80% relative humidity. *Magnaporthe oryzae* ZHONG-10-8-14 was cultured at 28°C on oatmeal agar medium (oatmeal, 50 g/liter, agar 30 g/liter) for 2 weeks. Conidia formation was then induced under light for 2 to 3 days. The spores were collected in sterile water and were used for inoculation.

Pathogen inoculation and DAB staining.

Plant pathogen inoculation assays were performed as described previously (Guo et al. 2011). For leaf inoculation assay, 2-week-old rice seedlings were used for *M. oryzae* infection. Conidial suspensions (1×10^5 to 5×10^5 conidia per milliliter in 0.2% Tween-20) was sprayed onto the leaves of rice plants, and then, the plants were incubated in darkness with 100% humidity; 24 h later, the plants were grown under the standard rice growth condition, as described above. For rice-sheath inoculation assays, the same concentration of conidial suspensions as spray inoculation were injected into inner leaf sheaths of rice seedlings, and then, the inoculated rice leaf sheaths were kept under 100% humidity until microscopic examination.

H₂O₂ accumulation was detected by DAB staining. The leaf sheaths inoculated with *M. oryzae* at indicated times were stained with 1% solution of DAB in Tris-HCl buffer (pH 6.5). After vacuum infiltration for 10 min, the samples were incubated at room temperature for around 20 h in darkness. When the brown spots appeared clearly, samples were bleached by boiling in ethanol/lactic acid/glycerol (1:1:1, vol/vol/vol) for 15 min. Images were captured using the light microscope (Olympus BX51).

qRT-PCR.

Rice total RNA was extracted by Trizol reagent, following the manufacture’s manual (Invitrogen). The extracted RNA was subjected to Dnase I digestion (Takara). cDNA was synthesized from 1 µg of total RNA, using M-MLV reverse transcription (Promega). All the qRT-PCR was performed on a CFX96TM

Fig. 7. Working model for *Magnaporthe oryzae* infection strategy. At early infection stage, *M. oryzae* activates host (rice) abscisic acid (ABA) and abiotic stress signaling to suppress immune responses, such as salicylic acid (SA)- and ethylene (ET)-mediated disease resistance. However, at the later infection stage, CK signaling is activated, which co-opts the plasma membrane-located transporters (rectangles) to regulate the nutrient efflux to feed massive growth of fungi in rice apoplast. In this progress, ABA and CK signaling are precisely regulated and sequentially activated at different infection stages. The small elliptic rings filled with lighter and darker shading represent sugars and other nutrients, respectively.

real-time system (Bio-Rad) with the SYBR green kit (Takara). The $2^{-\Delta\Delta CT}$ method was used for calculating the relative gene-expression levels. *OsActin2* or *ubq5* was used as the internal control. The primers used in this study are listed in Supplementary Table S3.

Immunoblotting.

The primary antibodies were purchased from Agrisera (Agrisera). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblotting were performed as previously described (Elmore et al. 2012). The following primary antibodies were used: rabbit anti-V-H⁺-ATPase (1:5,000), rabbit anti-catalase (1:1,000), hen anti-BiP2 (1:2,000), rabbit anti-H⁺-ATPase (1:3,000), and rabbit anti-VDAC1 (1:5,000). Secondary goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) (Cwbiotech) or goat anti-chicken IgY-HRP (Abbkine) conjugates were used at a concentration of 1:10,000 for detection, using enhanced chemiluminescence (Pierce).

PM enrichment and protein preparation.

PM purification and enrichment were performed using aqueous two-phase partitioning, as described previously with slight modification (Elmore et al. 2012). Briefly, rice leaves (20 g) were homogenized with a blender in 200 ml of ice-cold homogenization buffer containing 50 mM MOPS (pH 7.0), 0.33 M sucrose, 5 mM EDTA, 2 mM dithiothreitol (DTT), 1.5 mM ascorbate, 0.2% (wt/vol) insoluble polyvinylpyrrolidone, and 1 mM phenylmethylsulfonyl fluoride. Then, PM was purified from the microsomal fraction using aqueous polymer two-phase partitioning. The final upper phase fraction containing enriched PM vesicles were diluted with 35 ml of ice-cold H₂O and were centrifuged at 90,000 × g for 60 min. The PM pellets were frozen in liquid N₂ and were stored at -80°C until proteomic analysis.

To prepare the PM proteins for trypsin digestion and iTRAQ labeling, PM pellets were resuspended in 30 µl of 3× laemmli (without DTT) containing 6 M urea and were sonicated in a water bath. After centrifugation at 12,000 × g for 5 min, the supernatant was collected. Protein samples were quantified using a Pierce 660-nm protein assay kit with bovine serum albumin as the standard (Thermo Scientific).

Protein digestion and iTRAQ labeling.

Proteins for trypsin digestion were prepared according to the manufacture's manual (AB Sciex). In brief, each sample (100 µg) was reduced by 2 µl of reducing reagent at 37°C for 1 h, then, blocked by 1 µl of cysteine-blocking reagent at room temperature for 10 min. The protein solution was applied to a 10K ultrafiltration device (Sartorius) for in-filter digestion. The samples were centrifuged at 12,600 × g for 20 min and were then digested with 2.5 µg of trypsin in 50 µl of 100 mM TEAB (tetraethylammonium bromide), overnight, at 37°C. The peptide solution was collected at 12,600 × g centrifugation for 3 min and was further freeze-dried in a centrifugal vacuum concentrator. The peptides were resolved in 50 µl of 20 mM TEAB and were labeled by using iTRAQ 8-plex kits according to the manufacturer's manual (AB Sciex). All labeled samples were vacuum-dried and were stored at -80°C until LC-MS/MS analyses.

Off-line strong-cation exchange (SCX) fractionation of labeled peptides and LC-MS/MS analysis.

The iTRAQ labeled peptides were fractionated by using SCX chromatography on an Eksport ultraLC 100 system (AB Sciex). Peptide samples were reconstituted in buffer A (20 mM ammonium formate, pH 10) and elution of the peptides was achieved with a gradient of 5 to 90% buffer B (20 mM ammonium formate, 80% acetonitrile, pH 10) for 60 min, at a flow

rate of 1 ml/min. In total, 20 fractions were collected according to the peak area and were then desalted and lyophilized.

Each fraction was resuspended in 20 µl formic acid (0.1%) and was centrifuged at 12,600 × g for 10 min. The samples were separated on a Thermo Scientific EASY-Spray PepMap C18 column (75 µm × 15 cm, 2 µm) with a Thermo Scientific EASY-nLC 1000 HPLC (high-pressure liquid chromatography) system, and MS analyses were performed on a high-resolution Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific).

Protein identification and quantitative analysis.

Raw data were processed and searched against the Rice Genome Annotation Project (RGAP) database (version_7.0, 66,338 entries), assuming the digestion enzyme trypsin, using Mascot (Matrix Science; version 2.5.1). Mascot was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 parts per million. Max missed cleavages was 1.

Scaffold (version Scaffold_4.4.3, Proteome Software Inc.) was used to valid MS/MS-based peptide and protein identifications and to quantify iTRAQ label-based peptide quantification and protein identifications. Peptide identifications were accepted if they are established at greater than 95.0% probability by the Scaffold Local FDR (false discovery rate) algorithm. Protein identifications were accepted if they are established at greater than 5.0% probability to achieve an FDR of less than 1% and contain at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Channels were corrected according to the algorithm described by Shadforth et al. (2005). The reference channels were normalized to produce a 1:onefold change. All normalization calculations were performed, using medians to multiplicatively normalize data. The quality controls of three biological replicates were shown in Supplementary Fig. S9. Proteins with either log₂ fold change ≥0.5 or ≤-0.5 compared with the respective control and the *P* value ≤0.1 (Mann Whitney test) were considered to be differentially expressed.

Homology-based annotation and process over-representation analysis.

The homology-based annotation was performed by Blast2Go software to analyze the DEPs as previously described (Conesa et al. 2005). Briefly, all the protein sequences of DEPs were blasted (Blastp, version blast-2.2.28+) against the Swiss-Prot database with high E-value (1×10^{-10}) and number blast hits is 15. Then, the blast hit of each sequence was mapped with GO terms from the Gene Ontology database. Beijing Genome Institute's WEGO tool was used to detect GO biological process over-representation in the 24- and 48-hpi DEP groups, using all identified proteins as the background set. The most significant representative term from each cluster with *P* < 0.1 was reported.

The prediction of transmembrane helices in all identified proteins was performed through TMHMM software (2.0c version) on the Linux platform. The DEPs identified in this study were validated by RNA expression profile data from the Rice DB database. The genes with fold change ≥1.5 were considered to be differentially expressed.

RLKs prediction of rice *Oryza sativa* subsp. *japonica* cv. Nipponbare was performed using the data from Shiu et al. (2004) as raw data (1,235 RLK protein sequences were used for blast [Blastp, version blast-2.2.30+] against the RGAP database with a high E-value [1×10^{-20}]). Only the best-match proteins with high scores were selected as putative RLKs for the Nipponbare RLK dataset.

Apoplast metabolite analysis by GC-MS.

Apoplast metabolite analysis was performed according to the method described by O'Leary et al. (2014). Briefly, the leaf

samples were infiltrated with a 15-psi vacuum for 5 min followed by centrifugation at $15,000 \times g$ for 5 min, to collect AWF. Then, the AWF were vacuum-dried and used for the cytosol contamination assay and metabolite profiling analysis. The commercially available G6PDH and MDH assay kits (Sigma) were used for purity assessment of the AWF.

After the dried AWF was resolved by HPLC-grade water, ribitol (20 $\mu\text{g}/\text{ml}$) was added as an internal quantitative standard and the derivatization of samples by *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide was performed as previously described (Lisec et al. 2006). Samples were separated by a 30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm film thickness Rtx-5MS column (Restek) and were analyzed by using a GC-MS-QP2010 Ultra system (Shimadzu). The injection mode was splitless and the carrier gas was helium (1.2 ml/min). Mass spectra were equipped with electron impact ionization (70 eV), and the full scan range was 70 to 600 m/z .

Measurement of ABA, CK, and apoplast sugars by HPLC-MS/MS.

The ABA and transzeatin were determined according to previously described methods (Forcat et al. 2008). The rice leaf samples were ground in liquid nitrogen and were homogenized in 10% methanol containing 1% acetic acid. For measurement of ABA, $^2\text{H}_6$ -ABA was added as the internal standard. The MS was operated in negative mode and MRM for transitions m/z was used for analysis of $^2\text{H}_6$ -ABA (269 > 159) and endogenous ABA (263 > 153). The transzeatin was analyzed in positive mode and was used for analysis of transzeatin with MRM transitions m/z 220 > 136. External standard curves were used to quantify transzeatin levels. Samples were analyzed by a 1260 Agilent HPLC coupled with AB Sciex QTRAP 4500 mass spectrometer (MS) equipped with an electrospray interface. ABA and transzeatin were separated on a Synerg 4- μm Hydro-RP 80 $\text{\AA}$ LC column 150 $\times$ 2.0 mm (Phenomenex).

For measure of apoplast sugars, rice leaves were harvested after incubation with 200 μM transzeatin or distilled water for 6 h. The AWF was extracted and was analyzed by the same HPLC-MS/MS system with an Inertsil-NH₂ HPLC column (4.6 $\times$ 250 mm, 2.5 μm). The MS was operated in negative mode and MRM transitions m/z was used for analysis of fructose (179 > 89), glucose (179 > 89), and sucrose (314 > 89), respectively. External standard curves of the respective sugars were used for quantification.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Beijing Genomics Institute WEGO tool:
<http://wego.genomics.org.cn/cgi-bin/wego/index.pl>
 Rice DB's *Oryza sativa* database: <http://ricedb.plantenergy.uwa.edu.au>
 Rice Genome Annotation Project website: <http://rice.plantbiology.msu.edu>