RESEARCH ARTICLE

Proteomic analysis of rice plasma membrane reveals proteins involved in early defense response to bacterial blight

Fang Chen, Yuexing Yuan, Qun Li and Zuhua He

National Key laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences, Shanghai, China

Plant plasma membrane (PM) proteins play important roles in signal transduction during defense response to an attacking pathogen. By using an improved method of PM protein preparation and PM-bound green fluorescent protein fusion protein as a visible marker, we conducted PM proteomic analysis of the rice suspension cells expressing the disease resistance gene *Xa21*, to identify PM components involved in the early defense response to bacterial blight (*Xanthomonas oryzae* pv. *oryzae*). A total of 20 regulated protein spots were observed on 2-D gels of PM fractions at 12 and 24 h after pathogen inoculation, of which some were differentially regulated between the incompatible and compatible interactions mediated by *Xa21*, with good correlation between biological repeats. Eleven protein spots with predicted functions in plant defense were identified by MS/MS, including nine putative PM-associated proteins H^+-ATPase, protein phosphatase, hypersensitive-induced response protein (OsHIR1), prohibitin (OsPHB2), zinc finger and C2 domain protein, universal stress protein (USP), and heat shock protein. OsHIR1 was modified by the microbal challenge, leading to two differentially accumulated protein spots. Transcript analysis showed that most of the genes were also regulated at transcriptional levels. Our study would provide a starting point for functionality of PM proteins in the rice defense.

Received: October 20, 2005
Revised: December 6, 2006
Accepted: February 6, 2007

Keywords:
Defense response / Plasma membrane / Rice / *Xanthomonas oryzae* pv. *oryzae*

1 Introduction

Following the marvelous progress in plant genome and functional genomics, researchers now pay more attention toward the understanding of protein expression profile and protein–protein interactions (proteomics) either in a specific organ and development stage or during a particular biological process. Over the past years, great progress has been achieved in plant proteomics. These studies mainly focused on two catalogs, developmental proteomics of organs/tissues and organelles, and environmental proteomics in responses to biotic and abiotic stresses [1–4].

Rice, one of the most staple food crops, has been adopted as the model plant for monocot cereals, with its relative small genome sequenced [5]. This breakthrough achievement boosts research on the identification of function and regulation of rice proteins. As a powerful tool to systematically analyze the protein expression pattern in a given tissue of a certain development stage or condition, proteomics plays an important role in rice functional genomics of the post-genome era, along with a considerable amount of proteomic studies conducted with this crop. For example, proteomic studies of the embryo and endosperm development [6], anther [7], green and etiolated shoots [8], the basal region of

Correspondence: Professor Zuhua He, Institute of Plant Physiology and Ecology, SIBS, CAS, 300 Fenglin Road, Shanghai 200032, China
E-mail: zhhe@sibs.ac.cn
Fax: +86-21-54924015

Abbreviations: GFP, green fluorescent protein; **HIR,** hypersensitive-induced response; **MW,** molecular weight; **PHB,** prohibitin; **PID,** proliferation, ion, and death; **PM,** plasma membrane; **USP,** universal stress protein; **Xoo**, *Xanthomonas oryzae* pv. *oryzae*

developing seedlings [9], metabolic pathways in leaf, root and seed [10], and cultured suspension cells [11] have provided informative findings on rice protein profiles. More recently, the analysis of differential protein expression in rice leaves at the six different development stages allowed the identification of 49 differentially regulated protein spots [12]. Proteomics analysis was also employed to study rice responses to environmental stimuli, such as hormones including gibberellin, brassinolide, jasmonic acid and auxin [13–15], cold [16, 17], high temperature [18], drought [19], salinity [20, 21], wound [22], nitrogen nutrient [23], and ozone [24].

The plant response to pathogen attack or microbial signals has been a longtime interest of plant biologists and pathologists. Proteomics provides a direct evidence for target proteins involved in defense responses. For example, an *Arabidopsis* protein AtPhos43 and its homologues in tomato and rice are phosphorylated within minutes after treatment with flagellin or chitin fragments [4]. The analysis of leaf proteins of rice plants infected with blast fungus (*Magnaporthe grisea*) discovered 14 potential pathogenesis-related proteins that might be involved in the rice incompatible interaction with the pathogen [23]. Moreover, proteome analysis of the rice lesion-mimic *cdr2* mutant showed that a total of 37 proteins were differentially expressed between *cdr2* and wild type plants, with 28 up-regulated and 9 down-regulated in the *cdr2* mutant [25]. It was suggested that the programmed cell death in the *cdr2* mutant might be associated with active metabolic changes since many of these differentially expressed proteins were classified as metabolic enzymes [25].

Obviously, organelles-based proteomics will provide accurate and valuable information on protein compartmentalization and functionality [26–29]. Proteomic analysis of rice subcellular compartments has been carried out, including the plasma membrane (PM), vacuolar membrane, mitochondria, and chloroplasts [30]. PM is particularly important for all biological processes, given the fact that most of the receptor proteins for different signals are located in the PM, for example, proteomic analysis of the *Arabidopsis* PM identified components of transport, signal transduction, membrane trafficking, and stress responses [31]. However, there is not much information on direct PM proteomic investigation for defense response, mainly due to difficulty in PM protein analysis.

The rice receptor kinase XA21, predicted to be a PM protein, confers disease resistance to rice bacterial blight *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) [32], and autophosphorylation plays a role in the XA21 stability [33]. However, hitherto poor information is available about the XA21-mediated resistance. To gain an insight into rice PM proteins involved in the early defense response to *Xoo* particularly in the XA21-mediated resistance response, we conducted PM proteomic analysis using the *Xa21*-transgenic rice suspension cells inoculated with the compatible and incompatible *Xoo* races. We report here that a total of 20 protein spots out of 250–300 PM proteins were differentially regulated in the rice-*Xoo* interactions, of which 11 spots representing ten different proteins were determined with putative functions by MS/MS. We also observed that a hypersensitive-induced response (HIR) protein might be subjected to modification during the rice defense response. RT-PCR analysis validated regulation of the protein genes.

2 Materials and methods

2.1 Plant materials and *Xoo* inoculation

The *Xa21*-transgenic rice [32] suspension cells were cultured in liquid medium changed weekly, shaken in the dark at 120 rpm, 26°C. The suspension cells were coincubated with 10^7 cells/mL of the incompatible *Xoo* race 6 strain PXO99A (P6) and the compatible race 1 strain DY89031 (K1) as described in Ref. [34]. Cells were harvested at 0, 12, and 24 h after inoculation, followed by washing with ddH_2O to remove microbes. A constitutively expressing *Xa21-GFP* (green fluorescent protein) fusion driven by the 35S promoter was introduced into the same *japonica* variety TP309 to make suspension cells for PM detection.

2.2 Cell death detection

Cell death was detected to indicate the rice defense response stimulated by *Xoo* in the suspension cells. Inoculated cells were stained with 0.05% Evans blue for 15 min followed by washing with ddH_2O to remove excessive dye. Dyes taken up by dead cells were solubilized in 50% methanol with 1% SDS at 50°C for 30 min, and quantified by measuring the absorbance at 600 nm as described in Ref. [34]. Rice suspension cells were incubated at 80°C for 10 min as complete death control.

2.3 Isolation of PM fraction

Control and inoculated suspension cells were ground into powders in liquid nitrogen and resuspended in a solution containing 1.5 mM Tris-MES pH 7.8, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, 0.6% PVP w/v, 1 mM PMSF. After centrifuging at $80\,000 \times g$ for 30 min, the pellet was suspended in 5 mM phosphorous buffer, pH 7.8 with 0.1 mM DTT. The crude PM fraction was then further purified with the two-phase partition system containing 6.4% w/v PEG-3350 (Sigma), 6.4% w/v Dextran T-500 (Pharmacia), 0.25 M sucrose, 4.7 mM phosphorous buffer, pH 6.8. After being partitioned by centrifugation at $1000 \times g$, 5 min, repeated for three times, the up phases from three centrifugations were recovered and centrifuged at $120\,000 \times g$ for 30 min. The pellet was washed by ddH2O twice with centrifugation at $20\,000 \times g$, 4°C for 20 min, and then was suspended in the Cellular and Organelle Membrane Solubilizing Reagent (ProteoPrep Membrane Extraction kit, Sigma). Proteins were further separated according to the manufacturer's protocol

(Sigma). Preparation and protein analysis of PM samples were repeated biologically for three times with three technical repeats. Protein concentration was assessed with Bradford dye staining with reference to the standards [35].

2.4 Purity assessment of PM samples

PM sample was prepared from the transgenic suspension cells overexpressing *Xa21-GFP* fusion protein according to the method described above, without further protein extraction. PM purity was estimated by counting GFP-containing luminescent fragments of the PM preparation under the confocal laser microscope (Zeiss LSM510).

2.5 2-DE and detection of differentially expressed proteins

For protein detection, each of 100 μg PM protein sample was mixed with equal volumes of the rehydration buffer comprising 7 mM urea, 2 M thiourea, 0.5% Triton X-100, 0.5% IPG buffer (pH 4–7), 20 mM DTT, and bromophenol blue, and loaded onto IPG strips (linear pH 4–7, 17 cm, BioRad), kept at 17°C for 10 h, then followed by electrophoresis at 200 V for 2 h, 250 V for 30 min, 1000 V for 1 h, 10 000 V for 10 h, according to the manufacturer's protocol (BioRad). After electrophoresis, IPG strips were soaked in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue by shaking for equilibration for two times, the first with 2% DTT (10 min) and the second with 2.5% iodoacetamide (3 min). The second dimension electrophoresis was carried out on 12% SDS-PAGE at a constant current 60 mA for 5 h. Proteins were detected by silver stain [36], scanned, and analyzed for differentially expressed spots by the Melanie 4 software. For the preparation of target proteins, 250 μg PM protein sample was loaded.

2.6 Identification of proteins by MS/MS

Protein spots of interest were excised from two or three replicated gels. Briefly, silver-stained gel pieces were destained in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate at room temperature for 20 min. The pieces were then washed twice with ddH_2O and dehydrated in 100% ACN for 10 min, followed by drying completely at 37°C for 10 min. To conduct in-gel tryptic digestion, samples were incubated in a digestion mixture containing 20 mM ammonium bicarbonate and 12.5 ng/μL trypsin at 4°C for 30 min and then digested at 37°C overnight. After incubation twice in 50% ACN with 0.1% TFA for 30–40 min, trypsin-digested peptides were collected from the supernatants and dried under the protection of N_2 for subsequent MS identification [37].

Peptide samples were redissolved in 50% ACN, 0.1% TFA and 5 mg/mL CHCA (Sigma), then were loaded and air-dried on a target plate (Applied Biosystems). The mass range of PMF was scanned from 700 to 3200 Da by an ABI 4700 MALDI TOF-TOF proteomics analyzer (Applied Biosystems), followed by MS/MS analysis. Data were analyzed using the GPS (Applied Biosystems)/MASCOT software (Matrix Science) and searched against the protein database (http://www.ncbi.nlm.nih.gov) to retrieve the matched proteins.

2.7 Phylogenetic analysis of rice proliferation, ion, and death (PID) superfamily

Rice *PID* genes were searched from the whole rice genome (*japonica* Nipponbare) *via* the public GenBank database (http://www.ncbi.nlm.nih.gov/). The ORF regions of rice *PID* genes were multialigned by Megalign and ClustalX 1.81. Puzzle 5.2 and TreeView 1.6.1 were used to perform the phylogenetic analysis.

2.8 Gene expression analysis by RT-PCR

Total RNAs of the rice *Xa21*-transgenic suspension cells were extracted at 12 and 24 h after *Xoo* inoculation with mock inoculation as control, followed by the first cDNA strand synthesis for RT-PCR using SuperScriptTM RT-PCR kit (Invitrogen). RT-PCR primers were designed based on the cDNA sequences of the identified protein genes (see Table 1). The rice *ubiquitin-1* (*Ubi*) was used as the inner control.

Table 1. Primers used in RT-PCR

Protein	Forward primer	Reverse primer
2	5′ tccttccttcactctcccg 3′	5′ cggctataatactggtcaagg 3′
6	5′ gagatgaccaatggcggag 3′	5′ cacgctgtgtgtgatgaact 3′
8	5′ ctgcttcggtgctggttg 3′	5′ gctctatctggttgtgtcaa 3′
9	5′ ccgtcgagaagaagcaggt 3′	5′ aacccggcagcgttgagg 3′
10, 11	5′ gtgcaaactctgattgttgata 3′	5′ tcgaagtattgcgtaaccaga 3′
14	5′ cttcggtgcgcagatggg 3′	5′ gcctccttgtagtcttcaaa 3′
15	5′ gcctgtggagtgagcagag 3′	5′ gaagtacttcccctggtgga 3′
17	5′ tcgcagtcacaaatcctacaa 3′	5′ ggacgtgaacccactccag 3′
18	5′ atgatggaggcggtgcg 3′	5′ ggaggcggcttgacgac 3′
20	5′ gtgttcgacccttctccc 3′	5′ tggcgttgtcggggaggc 3′
Ubi	5′ gacggacgcaccctggctgactac 3′	5′ tgctgccaattaccatataccacgac 3′

3 Results

3.1 Defense activation in suspension cells by *Xoo*

XA21 is predicted to perceive the *Xoo* signal at the cell surface [32], leading to the "gene for gene" resistance response. To confirm that the *Xa21*-expressing suspension cells respond to *Xoo* in a race-specific pattern, we incubated the suspension cells with the incompatible strain P6 and the compatible strain K1 to measure induced cell death. Evans blue staining indicated that cell death occurred earlier and stronger in the incompatible interaction (Fig. 1), with signifi-

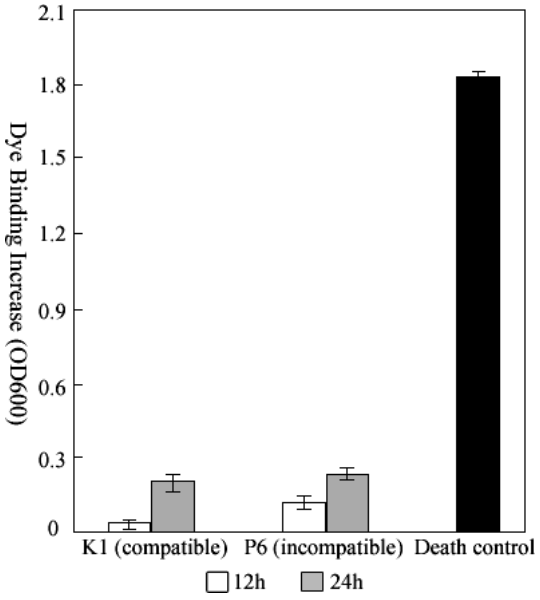


Figure 1. Cell death induced by *Xoo* in rice suspension cells. Values are mean ± SE of three replicates at 12 and 24 h after inoculation. Indicating that the defense response was activated by the incompatible strain P6 and compatible strain K1 in the cells. Killed cells by hot water as the complete death control.

cant difference in cell death between the two interactions at 12 h. Similar result was also observed in the previous experiments [34], indicating that rice defense response was activated in the suspension cells by the inoculation with *Xoo*. Because rice suspension cells grow as small calli, not single cells, cell death probably occurred only in epidermal cells during the early defense response as previously observed [34]. Most of the cells remained alive compared with the death control (Fig. 1). These results provide the foundation for our PM proteomics analysis focused on differentially expressed proteins during the early defense response.

3.2 Isolation and verification of PM compartment from rice suspension cells

We established an efficient and reliable PM protein preparation procedure by combining the two-phase partition method and the ProteoPrep membrane extraction kit. We observed a great improvement in protein separation by 2-DE using the procedure in comparison with the conventional two-phase partition protocol (data not shown).

Usually, the purity of PM is evaluated by determining the activity of the PM marker enzyme, H^+-ATPase [30, 38]. However, this ATPase maker method is somehow technically difficult for routine analysis. Other detection methods for PM preparation would be feasible such as proposed GFP fusion proteins to determine the visible localization of proteins in organelles [11]. Given that XA21 accumulates at low level with the native promoter, we used the *Xa21-GFP* over-expressing suspension cells to determine the purity of our PM fraction preparations. As expected, the PM sample of the *Xa21-GFP* cells displayed fluorescence on about 70% membrane debris under the fluorescent microscope (Fig. 2), and no any fluorescence was detected in the non-GFP transgenic PM fractions. Therefore, our preparation of PM samples contained at least 70% PM fraction, this reliable PM purity was the same as those obtained with methods used by other researchers [30, 38].

3.3 Differential expression of PM proteins in response to *Xoo*

We observed a total of about 250–300 visible protein spots on each 2-D gel with silver staining (Fig. 3), similar to the report by Tanaka *et al.* [30]. Based on biological and technical repeats, we systemically screened proteins that were differentially regulated in the early response to both the incompatible and compatible bacterial strains. According to the analytical results, good correlation was observed for whole images between the biological repeats (match rates were from 79.1 to 87.5%), and between technical repeats (match rates were over 90%), indicating reliable reproducibility of the experiments. Screening by the image software indicated potential differentially regulated spots at 12 and 24 h after inoculation (data not shown). We determined the regulated proteins based on the criterion: regulated spots could

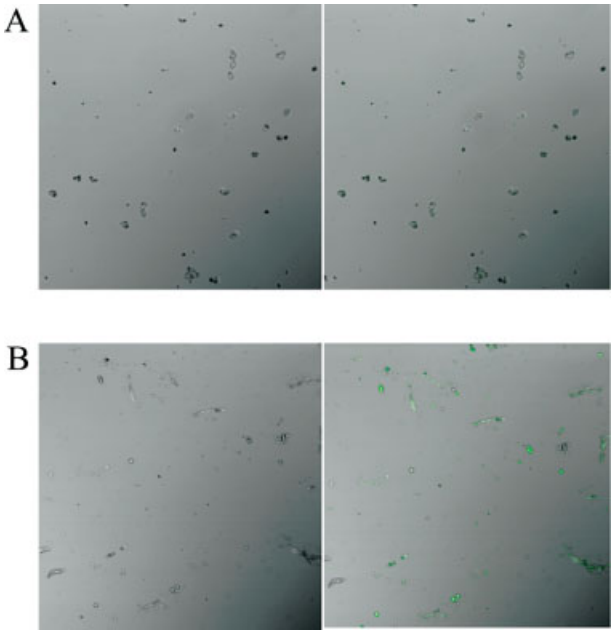


Figure 2. Detection of GFP-labeled rice PM by confocal microscopy. (A) Confocal observation of rice PM fragments from non-GFP-transgenic cell control. (B) Confocal observation of rice PM fragments cells overexpressing *Xa21*-GFP fusion protein, indicating that approximate 70% fragments of PM preparation were GFP-containing. Left panel, bright field image; right panel, a merged image in (A) and (B).

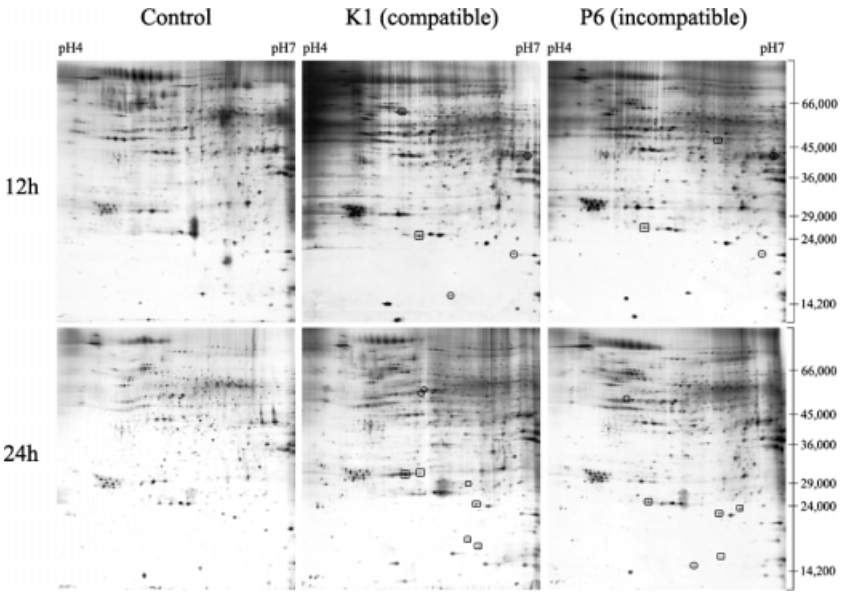


Figure 3. Representative 2-DE images of rice PM proteins at 12 and 24 h after *Xoo* inoculation. Three biological repeats were carried out for data analysis. The up-regulated spots are marked with squares, and down-regulated spots are marked with circles. Only those with a two- or above-fold change were treated as regulated proteins (see Fig. 4C). Protein MW markers and p*I* region are indicated.

be reproduced in all biological and technical experiments, and their levels (percentage volumes) changed at least two-fold, as shown in Fig. 3. Finally, we observed a total of 20 protein spots with reliable expression regulation in the early rice defense response to *Xoo* (Fig. 4A). These proteins were regulated differentially in the incompatible and compatible interactions (Table 2). There were three (spots 1, 7, 14) and six (spots 3, 4, 9–12), one (spot 5) and one (spot 15) spots regulated only in the compatible K1 inoculation at 12 and 24 h, in the incompatible P6 inoculation at 12 and 24 h, respectively. Two (spots 6, 17) and three (spots 8, 16, 19) spots were regulated in both P6 and K1 inoculations at 12 and 24 h, respectively. Spot 13 was up-regulated at 12 and 24 h only during the P6 inoculation. Spot 20 was down-regulated at 12 h in the K1 inoculation, and at 24 h in the P6 inoculation. Spot 2 was up-regulated at 12 and 24 h during the P6 inoculation, while it was up-regulated at 12 h but decreased to the basal level at 24 h during the K1 inoculation. Interestingly, spot 18 was down-regulated at 12 and 24 h by P6, but up-regulated at 24 h by K1, indicating that this protein could play an important role in the rice defense response to *Xoo*.

Table 2. Expression patterns of regulated putative rice PM proteins in response to *Xoo*

Protein	Description of expression pattern
1, 14	Up-regulated at 12 h in compatible interaction
2	Up-regulated at 12 h in compatible interaction Up-regulated at 12 and 24 h in incompatible interaction
3, 4, 11	Down-regulated at 24 h in compatible interaction
5	Up-regulated at 12 h in incompatible interaction
6, 17	Down-regulated at 12 h in both compatible and incompatible interactions
7	Down-regulated at 12 h in compatible interaction
8, 16, 19	Up-regulated at 24 h in both compatible and incompatible interactions
9, 10, 12	Up-regulated at 24 h in compatible interaction
13	Up-regulated at 12 and 24 h in incompatible interaction
15	Up-regulated at 24 h in incompatible interaction
18	Down-regulated at 12 and 24 h in incompatible interaction Up-regulated at 24 h in compatible interaction
20	Down-regulated at 24 h in incompatible interaction Down-regulated at 12 h in compatible interaction

3.4 Identification of regulated PM proteins

PM proteins were identified by MS/MS (Fig. 5), since the Edman sequencing method does not work well for membrane proteins [30]. Of 20 regulated spots, 11 were successfully identified by MS/MS with putative functions (Fig. 4B), others either could not be determined by MS/MS, or matched proteins without any predicated functions (data not shown). Statistical evaluation showed their relative volumes with standard errors (Table 3), indicating again their reliable differential expression. The predicted molecular weights (MW) and p*I* of these proteins were consistent with their positions on the gel (Fig. 4A, Table 4). Spots 10 and 11 were identified to be the same protein (Table 4). Particularly interesting, protein spots 2 and 9 were induced more than five-fold by *Xoo* (Fig. 4C).

Among the other ten regulated proteins, six proteins, H^+-ATPase, protein phosphatase, prohibitin (OsPHB2), HIR protein (OsHIR1), quinone reductase, zinc finger, and C2 domain protein are considered as putative PM-associated proteins [39–42]. The universal stress protein (USP) was suggested to be a membrane-associated protein since the *E. coli* UspA may have a role in fatty acid/membrane lipid metabolism [43]. Low molecular weight (LMW) HSPs have been

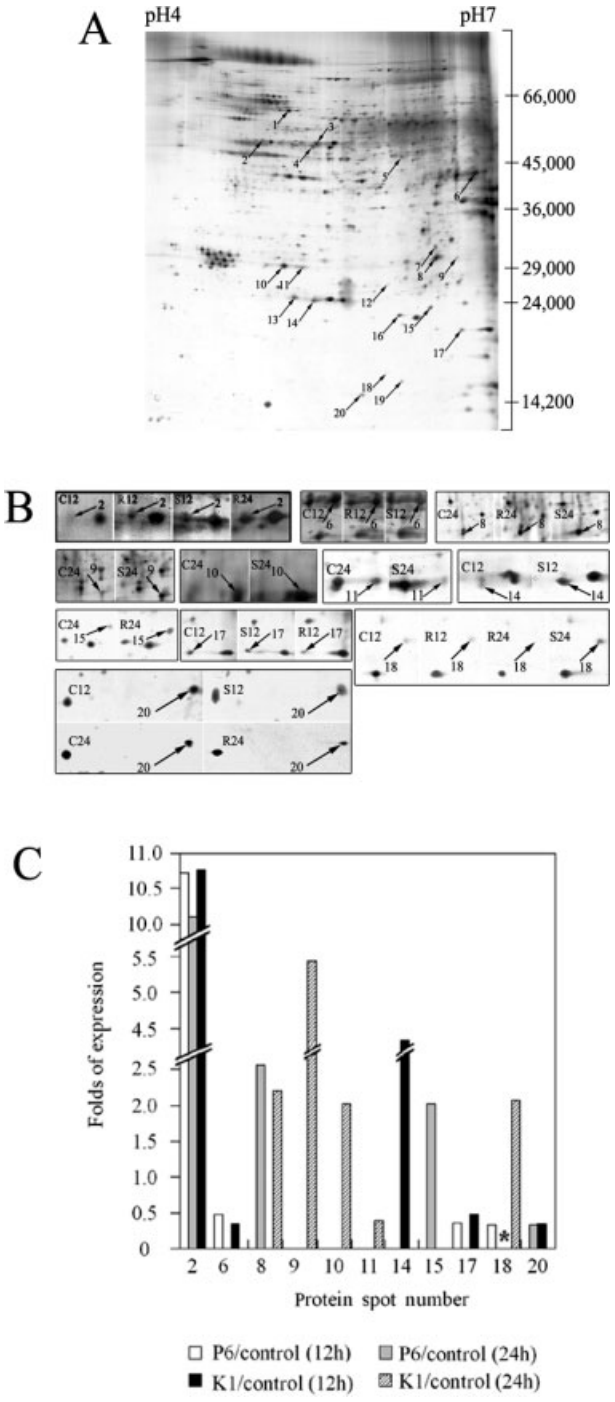


Figure 4. Expression patterns of differentially regulated spots. (A) A total of 20 spots were regulated in the early defense response. (B) Profiles of 11 differently regulated proteins that were successfully identified by MS/MS. C, control; R, resistant (incompatible) reaction to the strain P6; S, susceptible (compatible) reaction to the strain K1; 12 and 24, 12 and 24 h for treatment. (C) Relative abundance of the 11 proteins in comparison with the controls that were arbitrarily set as 1, as quantified by the Melanie 4 software. Only those with two- or above-fold changes are shown.*Protein 18 was completely suppressed at 24 h in P6 inoculation.

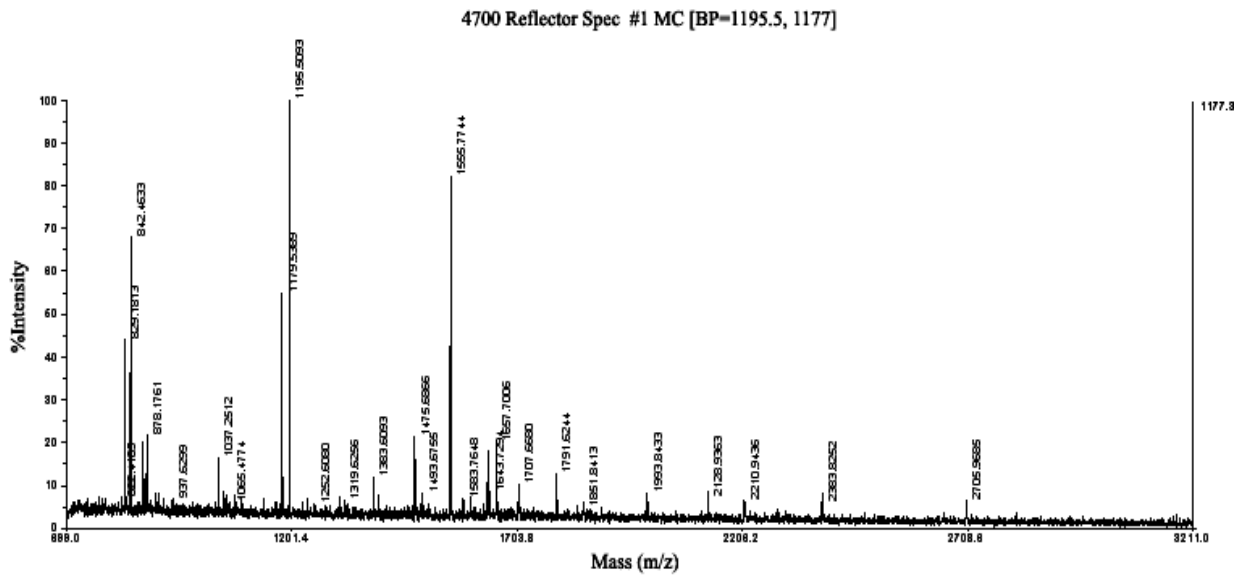
observed in different subcellular compartments, it might also associate with PM as the yeast LMW Hsp12 [44]. Ascorbate peroxidase and alcohol dehydrogenase 1 (ADH1) are probably not located on the PM (Table 4). Ascorbate peroxidase is a mitochondrial membrane-bound protein, and ADH1 might be a cytoplasmic protein, which could be co-isolated with PM fraction. All these proteins including ascorbate peroxidase and ADH1 have been repeatedly identified as defense or stress response-regulated proteins. The further functional characterization would provide insight into their roles in the rice defense response.

Interestingly, we identified spots 10 and 11 as the same OsHIR1 protein with different p*I*s (Table 4), suggesting that this protein might be subjected to phosphorylation-regulation or other kind of modification during the defense response, given the fact that this protein contains five potential phosphorylation sites (Fig. 6A). Similar result is also observed in an HIR-related protein, OsPHB1, in a rice cell death mutant [45]. Our results suggest that HIR proteins might actively function in plant defense. PHB and other HIR proteins containing the PHB domain belong to a protein superfamily PID involved in development and defense responses [46]. As shown in Fig. 6A, OsHIR1 shared sequence or structural similarity with ZmHIR1, OsPHB1, OsPHB2, and ZmPHB2. A total of 18 *PID* genes were mined from the *japonica* rice genome, and a phylogenetic tree was established (Fig. 6B). It was shown that there were eight OsHIR and seven OsPHB proteins in the rice genome. Other three proteins (GenBank accession nos. AAT85034, AAP53873, and BAC99654) were classified independently.

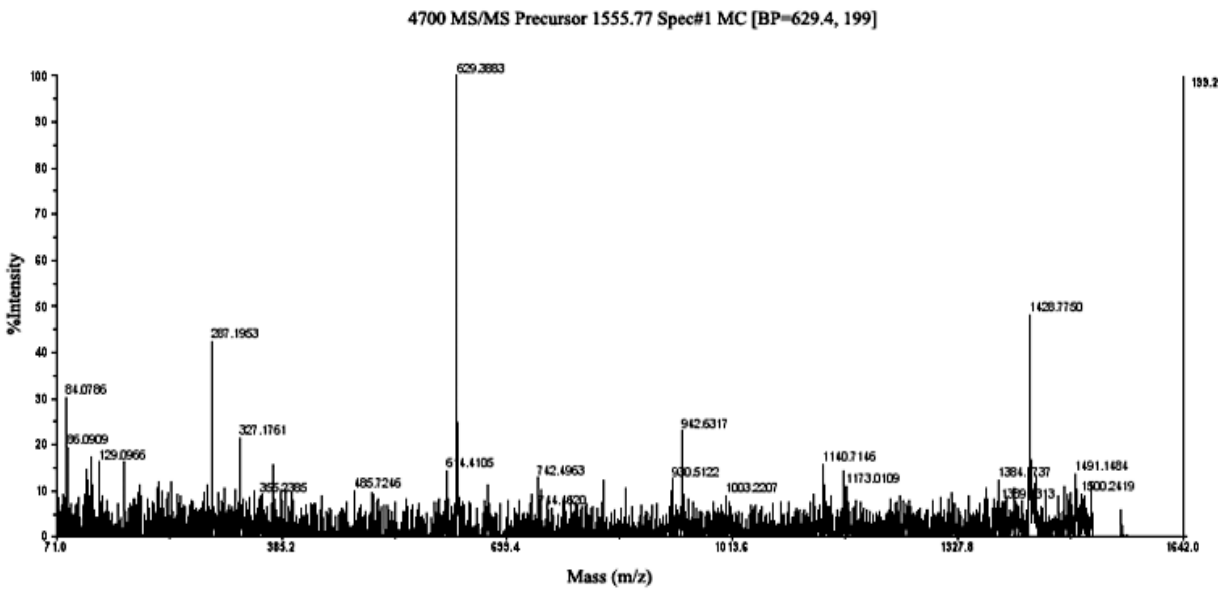
3.5 Differential expression of regulated protein genes

In order to confirm transcriptional regulation of these genes, we detected their transcripts by RT-PCR. As shown in Fig. 7, five genes, *ADH1, OsHIR1* (spot 10), *ascorbate peroxidase, quinone reductase,* and *zinc finger and C2 domain protein-like* showed good correlation between transcript and protein levels at the time points/interactions indicated in Table 2. Transcription levels of H^+*-ATPase, USP,* and *LMW HSP* also exhibited good correction at some time points with protein levels (Fig. 7, Table 2), except transcription levels of H^+*-ATPase* in resistant reaction, 24 h (R24), *USP* in susceptible reaction, 24 h (S24), and *LMW HSP* in S12 were not consistent with corresponding protein levels. Similarly, transcripts of *ADH1, OsHIR1, ascorbate peroxidase, quinone reductase,* and *zinc finger* and *C2 domain protein-like* displayed to be regulated, while protein levels were not altered in some interactions (Table 2). Interestingly, protein phosphatase was shown to be up-regulated at R24 and S24, however, its transcript was not, which instead was significantly induced at R12 and S12. This gene might also be subjected to regulation by mock inoculation conditions, given that its control at 24 h (C24) had higher signal than C12. Also similar were the controls for *zinc finger* and *C2 domain protein-like* (Fig. 7). *OsPHB* seemed to be slightly up-regulated in S24 but

A



B



C

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence
1057.5312	1057.4917	-0.0395	-37	60	68	ETPEAHVFK
1195.5701	1195.5066	-0.0635	-53	43	55	GASSETAAFAGAR
1252.6896	1252.609	-0.0806	-64	122	132	LPDNAKPEQIK
1555.859	1555.7734	-0.0856	-55	120	132	FRLPDNAKPEQIK
1599.8165	1599.7202	-0.0963	-60	56	68	IDWKETPEAHVFK
1643.8234	1643.7281	-0.0953	-58	81	95	VEVDDGNILQISGER
2129.072	2128.937	-0.135	-63	77	95	EEVKVEVDDGNILQISGER

Figure 5. Identification of protein 20 by MS/MS. (A) PMF resulting from MS analysis. Spectral peaks with masses of 1195 and 1555 were the two most abundant peptide fragments, and were selected for MS/MS analysis. (B) MS/MS result of mass-1555 peptide as an example. (C) Amino acid sequences of peptide fragments were obtained by PMF searching in database.

Table 3. The relative volume of regulated proteins

Protein	Relative volume (%)[a]					
	C12	R12	S12	C24	R24	S24
2	0.036 ± 0.008[b]	0.368 ± 0.034	0.390 ± 0.045	0.034 ± 0.008	0.364 ± 0.064	/[c]
6	1.972 ± 0.089	0.969 ± 0.070	0.659 ± 0.063	/	/	/
8	/	/	/	0.404 ± 0.044	1.036 ± 0.045	0.897 ± 0.099
9	/	/	/	0.108 ± 0.020	/	0.589 ± 0.077
10	/	/	/	1.213 ± 0.074	/	2.464 ± 0.725
11	/	/	/	0.357 ± 0.032	/	0.189 ± 0.012
14	0.159 ± 0.017	/	0.687 ± 0.052	/	/	/
15	/		/	0.191 ± 0.020	0.439 ± 0.022	/
17	0.396 ± 0.025	0.143 ± 0.033	0.188 ± 0.024	/	/	/
18	0.199 ± 0.009	0.066 ± 0.012	/	0.189 ± 0.015	ND	0.394 ± 0.073
20	1.065 ± 0.049	/	0.366 ± 0.030	0.967 ± 0.071	0.305 ± 0.030	/

a) Individual spot volume is shown as a percentage of the total spot volumes present in one gel.
b) SDs were derived from three independent biological repeats.
c) /, No apparent expression changes compared with corresponding control volumes.
C, Control; R, resistant (incompatible); S, susceptible (compatible); 12 and 24, 12 and 24 h after inoculation; ND, not determined due to too low abundance.

Table 4. Identification of regulated proteins by MS/MS

Protein	MW (Da)[a]	p*I*[b]	Identity	Accession number
2	54 245.6	5.03	H+-ATPase	NP_916591
6	41 566.9		Alcohol dehydrogenase 1	CAA34363
8	20 594.2	5.85	Protein phosphatase	XP_472680
9	30 783.2	6.99	PHB	XP_472766
10, 11	31 501.2	5.22	HIR protein	AAK54610
14	27 138.7	5.42	Ascorbate peroxidase	XP_470658
15	21 690.8	6.06	Quinone reductase	NP_916411
17	18 905.1	6.51	Zinc finger and C2 domain protein-like	XP_478243
18	18 869.5	5.61		AAP53941
20	17 899.1	5.8	LMW HSP	NP_912354

a,b) MW and p*I* were predicted by MS/MS results.

significantly down-regulated in R12 and R24, while the protein level was not altered in the resistant reactions (Fig. 7, Table 2). These results indicate that some of the genes were regulated at both transcription and protein levels, and a few were subjected to post-transcriptional regulation during the rice–*Xoo* interaction. Similar results were also observed in other reports [17, 47, 48].

4 Discussion

PM proteomics analysis has been thought particularly difficult because this subcellular compartment contains a large amount of lipids and saccharides, and many PM proteins contain hydrophobic peptides. In this paper, we have adopted a method to isolate PM proteins by combining the conventional two-phase partition method and the membrane extraction kit, to facilitate PM protein preparation and improve the quality of 2-DE of PM proteins, which showed good reproducibility among independent experiments. We also used a PM-bound GFP fusion protein to monitor the purity of PM preparation, which exhibited visible proportion of PM fragments in the membrane pellet (Fig. 2). These methods would certainly further enhance the effectiveness of PM proteomics study. We used silver staining to detect differentially expressed PM proteins in considering low abundance of many PM proteins. However, silver staining probably affected the efficiency of MS/MS, about 60% tested proteins were successfully identified by MS/MS in our current study (Tables 2 and 4).

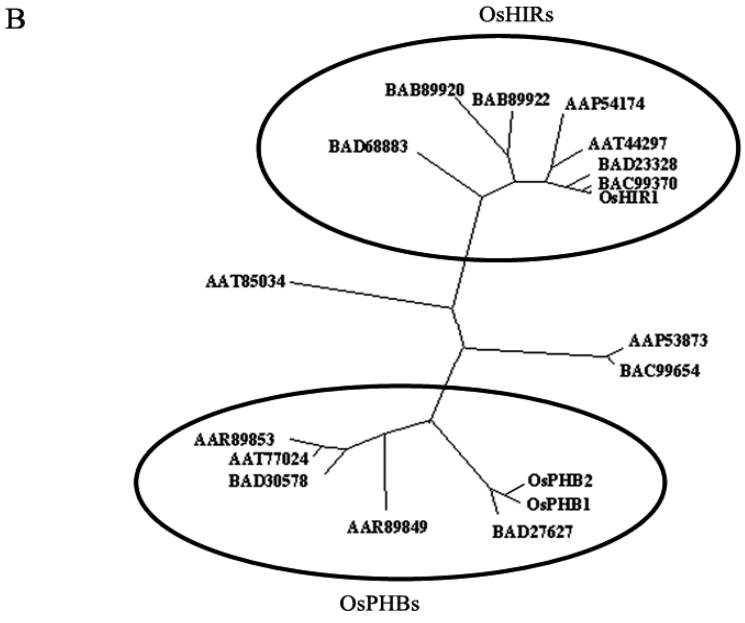


Figure 6. Sequence alignment of OsHIR1 and related proteins and phylogenetic tree of the rice PID family. (A) Multiple alignments of amino acid sequences of OsHIR1, OsPHB2, and homologous proteins, OsPHB1 from rice, ZmHIR1 (AF236373) and ZmPHB2 (AF236369) from maize, showing structural similarity between HIR and PHB proteins. The PHB domain is indicated by box. Amino acids underlined are predicted phosphorylation sites with *for putative phosphorylated residues. (B) An unrooted phylogenetic tree showing the relationship of 18 members of the rice PID superfamily.

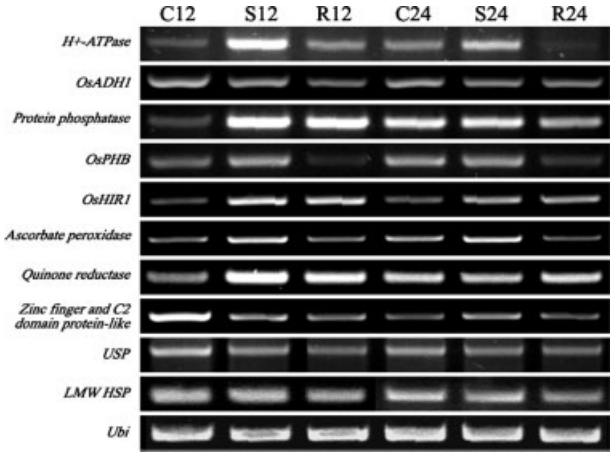


Figure 7. RT-PCR detection of gene expression. RT-PCR was performed using the primers (Table 1) with 25 cycles, and *Ubi* as the inner control. C, control; S, susceptible (compatible); R, resistant (incompatible); 12 and 24, 12 and 24 h after treatment.

Plant PM proteomics study has been conducted with the two model plants, *Arabidopsis* [31] and rice [30]. Among hundreds of rice PM proteins, seven have been identified by the Edman sequencing strategy and 51 by MS/MS [30]. We reported here for the first time using proteomic approach for the identification of PM proteins involved in the rice defense response to the bacterial blight. The rice suspension cells expressing XA21 responded to the pathogen strains (Fig. 1), suggesting that race-specific and basal defense responses were stimulated through signal perception at the cell surface and transmembrane-transmission by the membrane receptor and other PM-associated components [32–34]. We postulated that these PM-associated components could be regulated in the rice cell during the early defense response.

In this study, we observed a total of 20 protein spots regulated by the pathogen challenge (Figs. 3 and 4) and identified at least eight putative PM-associated proteins with potential functions in rice defense (Fig. 4, Table 4). Both H^+-ATPase and quinone reductase, two enzymes functioning in

electron and energy transfer, were up-regulated in the rice-*Xoo* interactions (Fig. 4C, Table 2). H^+-ATPase has been recognized as the proton pump to stimulate the hypersensitive response during plant defense responses [49]. In rice, this protein has not been found to be involved in defense response. Our study suggests that the rice H^+-ATPase might play a similar function as a proton pump in defense. Quinone reductases are detoxification enzymes, possibly function in protecting against pathogen-induced oxidative stress, as suggested for the auxin-responsive quinone reductase [50]. Interestingly, we observed that some defense-related proteins including HIR, PHB, and USP were regulated in the rice defense response, and that OsHIR1 (spot 10) and OsPHB2 (spot 9) exhibited similar expression pattern (Fig. 4, Table 2). Although further studies are required to verify the importance of these proteins in the rice defense, the preliminary result would serve as a starting point for functionally characterizing these proteins and their family (Fig. 6B).

A protein phosphatase was also up-regulated, indicating that phosphorylation event could play a role in the early defense response. In support of this hypothesis, we observed that OsHIR1 might be differentially modified or phosphorylated during the pathogen challenge, leading to the accumulation of probably phosphorylated OsHIR1 (Fig. 4B and C, Table 2). Similarly, OsPHB1 was also found to be hyperphosphorylated in the rice *cdr1* lesion-mimic mutant after the treatment of calyculin A, an inhibitor of protein phosphatase [51]. It would be interesting to investigate whether the phosphatase directly reacts with OsHIR1, and whether mutations on the potential phosphorylation sites would abolish the function (if any) of OsHIR1.

There were cell death events occurring during pathogen infection (Fig. 1). There could be more PM proteins regulated because of PM structure and permeability changes during infection. However, only 20 protein spots were found to be regulated in this defense response with the current techniques, of which ten proteins were already regulated at 12 h (Fig. 4, Table 2). We do not know how the whole PM components change during defense response. Hitherto not many PM proteins or genes (except those R receptor proteins) have been identified involved in defense, probably because most of the defense activities occur intracellularly or PM proteins are at too low abundance to be detected. Another possibility is that rice suspension cells grow as small calli not single cells, cell death occurs only in the epidermal cells of calli as observed (Fig. 1), resulting in low ratio of dead cell PM in preparation. Some advanced technology such as laser capture microdissection (LCM) could provide a more precious tool for further investigation of cellular events during the defense response. It has been frequently documented that protein level is not always consistent with transcript level [17, 47, 48]. Similarly, our experiment showed that transcript levels of some genes did not fit well with protein patterns revealed by 2-D image (Figs. 4 and 7, Table 2) Therefore, some proteins were regulated at the translation or post-translation level.

In summary, we found that a combining utilization of the two-phase partition method and the commercial membrane extraction kit could generate good quality 2-DE of PM proteins. We also indicated that a PM-bound GFP fusion protein could visibly monitor the purity of PM routine preparation. Our current study demonstrates that direct proteomics analysis of rice PM can be applied to identify potential PM components involved in the rice defense response to microbes. Some of the PM proteins identified in this report might play important roles in plant disease resistance, although further studies are required to verify the detailed localization and regulation of these proteins.

We would like to thank Weining Sun for critical reading of the manuscript, and Pamela Ronald for providing the Xa21 transgenic rice. This work was supported by the grants from National Natural Science Foundation of China (30125030 and 30421001), National Basic Research Program of China (2006CB101900).

5 References

[1] Agrawal, G. K., Rakwal, R., *Mass Spectrom. Rev.* 2006, *25*, 1–53.

[2] Hajheidari, M., Abdollahian, N. M., Askari, H., Heidari, M. *et al.*, *Proteomics* 2005, *5*, 950–960.

[3] Park, O. K., *J. Biochem. Mol. Biol.* 2004, *37*, 133–138.

[4] Peck, S. C., Nuhse, T. S., Hess, D., Iglesias, A. *et al.*, *Plant Cell* 2001, *13*, 1467–1475.

[5] International Rice Genome Sequencing Project, *Nature* 2005, *436*, 793–800.

[6] Komatsu, S., Kajiwara, H., Hirano, H.,*Theor. Appl. Genet.* 1993, *86*, 935–942.

[7] Imin, N., Kerim, T., Weinman, J. J., Rolfe, B. G., *Proteomics* 2001, *1*, 1149–1161.

[8] Komatsu, S., Muhammad, A., Rakwal, R., *Electrophoresis* 1999, *20*, 630–636.

[9] Tanaka, N., Mitsui, S., Nobori, H., Yanagi, K., Komatsu, S., *Mol. Cell. Proteomics* 2005, *4*, 796–808.

[10] Koller, A., Washburn, M. P., Lange, B. M., Andon, N. L. *et al.*, *Proc. Natl. Acad. Sci. USA* 2002, *99*, 11969–11974.

[11] Komatsu, S., Rakwal, R., Li, Z., *Plant Cell Tissue Organ Cult.* 1999, *55*, 183–192.

[12] Zhao, C., Wang, J., Cao, M., Zhao, K. *et al.*, *Proteomics* 2005, *5*, 961–972.

[13] Yang, G., Komatsu, S., *Genomics Proteomics Bioinformatics* 2004, *2*, 77–83.

[14] Rakwal, R., Komatsu, S., *Electrophoresis* 2000, *21*, 2492–2500.

[15] Casimaro, I., Marchant, A., Bhalerao, R. P., Beeckman, T. *et al.*, *Plant Cell* 2001, *13*, 843–852.

[16] Garbalino, J. E., Hurkman, W. J., Tanaka, C. K., Dupont, F. N., *Plant Physiol.* 1991, *95*, 1219–1228.

[17] Yan, S. P., Zhang, Q. Y., Tang, Z. C., Su, W. A., Sun, W. N., *Mol. Cell. Proteomics* 2006, *5*, 484–496.

[18] Lin, S. K., Chang, M. C., Tsai, Y. G., Lur, H. S., *Proteomics* 2005, *5*, 2140–2156.

[19] Salekdeh, G. H., Siopongco, J., Ghareyazie, B., Bennett, J., *Proteomics* 2002, *2*, 1131–1145.

[20] Abbasi, F. M., Komatsu, S., *Proteomics* 2004, *4*, 2072–2081.

[21] Yan, S., Tang, Z., Su, W., Sun, W., *Proteomics* 2005, *5*, 235–244.

[22] Shen, S., Jing, Y., Kuang, T., *Proteomics* 2003, *3*, 527–535.

[23] Konishi, H., Ishiguro, K., Komatsu, S., *Proteomics* 2001, *1*, 1162–1171.

[24] Agrawal, G. K., Rakwal, R., Yonekura, M., Kubo, A., Saji, H., *Proteomics* 2002, *2*, 974–959.

[25] Tsunezuka, H., Fujiwara, M., Kawasaki, T., Shimamoto, K., *Mol. Plant Microbe. Interact.* 2005, *18*, 52–59.

[26] Khan, M. M., Komatsu, S., *Phytochemistry* 2004, *65*, 1671–1681.

[27] Baginsky, S., Gruissem, W., *J. Exp. Bot.* 2004, *55*, 1213–1220.

[28] Warnock, D. E., Fahy, E., Taylor, S. W., *Mass Spectrom. Rev.* 2004, *23*, 259–280.

[29] Ephritikhine, G., Ferro, M., Rolland, N., *Plant Physiol. Biochem.* 2004, *42*, 943–962.

[30] Tanaka, N., Fujita, M., Handa, H., Murayama, S. *et al.*, *Mol. Genet. Genomics* 2004, *271*, 566–576.

[31] Alexandersson, E., Saalbach, G., Larsson, C., Kjellbom, P., *Plant Cell Physiol.* 2004, *45*, 1543–1556.

[32] Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S. *et al.*, *Science* 1995, *270*, 1804–1806.

[33] Xu, W. H., Wang, Y. S., Liu, G. Z., Chen, X. H. *et al.*, *Plant J.* 2006, *45*, 740–751.

[34] He, Z., Wang, Z. Y., Li, J., Zhu, Q. *et al.*, *Science* 2000, *288*, 2360–2363.

[35] Bradford, M., *Anal. Biochem.* 1976, *72*, 248–254.

[36] Blum, H., Beier, H., Gross, H. J., *Electrophoresis* 1987, *8*, 93–99.

[37] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., *Anal. Chem.* 1996, *68*, 850–858.

[38] Veronique, S., Joelle, V., Delphine, P., Nicolas, S.,,Christophe, M.,,*Biochem. J.* 2003, *373*, 289–296.

[39] Lindberg, S., Banas, A., Stymne, S., *Plant Physiol. Biochem.* 2005, *43*, 261–268.

[40] Roblin, G., Sakr, S., Bonmort, J., Delrot, S., *FEBS Lett.* 1998, *424*, 165–168.

[41] Nadimpalli, R., Yalpani, N., Johal, G. S., Simmons, C. R., *J. Biol. Chem.* 2000, *275*, 29579–29586.

[42] Serrano, A., Villalba, J. M., Gonzalez, J. A., Navas, P., Cordoba, F., *Biochem. Mol. Biol. Int.* 1994, *32*, 841–849.

[43] Jensen, R. B., Lykke, K., Frandsen, G. I., Nielsen, H. B. *et al.*, *Plant Mol. Biol.* 2000, *44*, 799–814.

[44] Farewell, A., Diez, A. A., Dirusso, C. C., Nystrom, T., *J. Bacteriol.* 1996, *178*, 6443–6450.

[45] Sales, K., Brandt, W., Rumbak, E., Lindsey, G., *Biochim. Biophys. Acta* 2000, *1463*, 267–278.

[46] Hakozaki, H., Endo, M., Masuko, H., Park, J. I. *et al.*, *Genes Genet. Syst.* 2004, *79*, 307–310.

[47] Gygi, S. P., Rochon, Y., Franza, B. R., Aebersold, R., *Mol. Cell. Biol.* 1999, *19*, 1720–1730.

[48] MacKay, V. L., Li, X. H., Flory, M. R., Turcott, E. *et al.*, *Mol. Cell. Proteomics* 2004, *3*, 478–489.

[49] Finni, C., Andersen, C. H., Borch, J., Gjetting, S. *et al.*, *Plant Mol. Biol.* 2002, *49*, 137–147.

[50] Laskowski, M. J., Dreher, K. A., Gehring, M. A., Abel, S. *et al.*, *Plant Physiol.* 2002, *128*, 578–590.

[51] Takahashi, A., Kawasaki, T., Wong, H. L., Suharsono, U. *et al.*, *Plant Physiol.* 2003, *132*, 1861–1869.

