Development of proteomic technology of shotgun and label free combined with multiple reaction monitoring to simultaneously detect southern rice black-streaked dwarf virus and rice ragged stunt virus

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Abstract The co-infection of rice caused by southern rice black-streaked dwarf virus (SRBSDV) and rice ragged stunt virus (RRSV) was widely found at many regions, such as Yunnan Province, China, and North and Central Vietnam. These rice viral diseases lead to seriously yield loss of rice. In this study, the proteomics technology of shotgun and label free combined with multiple reaction monitoring (MRM) was developed to detect rice sample of a single or/and co-infection. The shotgun assay indicated that some proteins coded by SRBSDV and RRSV were detected via the mode of in-gel digestion, except for P5-2, P7-2 and P9-2 of SRBSDV and P4b, P5, P6, P8a and P8b of RRSV. The technology of label free combined with MRM indicated that P2, P5-1, P4, P8, P7-1, P6, P9-1 and P10 of SRBSDV and P1, P3 and P9 of RRSV were higher abundance in rice plant, and P5-2, P7-2 and P9-2 of SRBSDV and P4b and P5 of RRSV were lower abundance in viruliferous-rice plant. So SRBSDV P9-1 and RRSV P3 was selected as marker molecule to be used in detection

technology, and the label free combined with MRM technology was established to detect two kinds of rice virus.

Keywords SRBSDV · RRSV · Shotgun protein proteomic · Multi reaction monitoring · Label free · Bio-informatics

Introduction

Over the last few years, southern rice black-streaked dwarf virus (SRBSDV), a *Fijivirus* species in the family *Reoviridae*, has infected rice and corn in many areas of PR China, Vietnam, Japan and Korea [9, 20, 27, 34, 35]. The infected rice was shown some symptoms of stunting, dark green leaf, white waxy or black-streaked swellings on stem, and brown and fragile roots [35]. In addition, the infected rice in seeding stage can lead to die, and there was litter production or non-production of rice at harvest time [35]. White black plant-hopper (WBPH, *Sogatella furcifera*) was transmitted vector of SRBSDV by transmission mode [22, 35]. The viral genomes contain ten linear genomic segments (namely S1–S10) [34, 35]. P6 protein was seem to be silencing suppressor [17], and P7-1 protein can induce the formation of tubular structures and is conferred to the function of virus movement [14]. The interaction of proteins were found between P6 and P5-1 using yeast two-hybrid (YTH) assay and bimolecular fluorescence complement (BiFC) assay [13]. In additional, the primary metabolism and the ubiquitin–proteasome of WPBH was disturbed by SRBSDV using de novo transcriptome assembling and massive parallel pyrosequencing [31].

Rice ragged stunt virus (RRSV), a member belong to the genus *Oryzavirus* in the family *Reoviridae*, can damage rice and corn in China and Vietnam in 2010–2012 [3, 6, 9,

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11, 15]. The viral genome consist of ten linear genomic segments (namely S1–S10), which encoded at least seven structural protein of P1, P2, P3, P4a, P5, P8b and P9, and three non-structural proteins of NS6, NS7 and NS10 [4, 11, 23–25, 29, 30]. Infected rice were appeared typical stunting, vein swelling or galls existed on the underside of leaf blades and outer surface of leaf sheaths, darkening of leaves and twisted with notches, slender vein in dorsal leaves. The infected rice at rice seeding was easily to be die or significant yield reduction [8]. Brown plant-hopper (BPH, *Nilaparvata lugens*) can transmit RRSV by a persistent manner, but can't transmitted via eggs [7]. In recent years, the co-infection of rice about SRBSDV and RRSV was found at many regions, such as Northern Vietnam, and Baoshan and Dehong in Yunnan Province, and Chongzuo and Nanning in Guangxi Zhuang Autonomous Region, PR China [3, 9, 11, 15]. The behavior mechanism of the co-infection in rice was thought to be volatile substance of the infected rice, which may be a role to induce planthopper [28]. Aiming to the detection methods about single infection, duplex RT-PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP) and Dot-immunobinding assay (DIBA) was develop to detect SRBSDV or RRSV from plant and vector insect [1, 5, 16, 27, 33, 36–38]. In present study, a shotgun proteomics was firstly detected many proteins coded by viral genome and label free technology combined with MRM was used to study the abundance level of protein, the label free combined with MRM technology was established to simultaneously detect SRBSDV and RRSV from rice plant against the target protein of SRBSDV P9-1 and RRSV P3.

Materials and methods

Methods design for detection rice virus

For detect SRBSDV and RRSV from viruliferous rice sample, the shotgun assay and label free combined with MRM was used in two detection methods (Fig. 1). Firstly, in the shotgun assay, the total protein from rice sample was separated using SDS-PAGE, then the gel was cut and digested to crude peptides using trypsin. The crude peptides were identified using the methods of protein identification by LC–MS/MS, and the data was searched by Protein pilot software (Version 4.0.8085). The results of shotgun assay indicated which protein coded by SRBSDV and RRSV belong to higher abundance or lower abundance (Fig. 1a). Secondly, because the rate of viral protein to the total protein is very low in rice sample, the few viral peptides eluted from analytical column may be not reached at ion trap after passing triple quadrupole. MRM approach can priority select the specific the precursor ions (m/z), let

it into ion trap and be analyzed. So in the label free assay, the viral protein with higher abundance was calculated the information of the precursor ions and the product ion using Skyline software (version 1.4.0.4421), the total protein from rice sample was digested, and the crude peptides were identified using LC–MS/MS in the MS method introduced the information of the precursor ions. The values of the precursor ions were input into the method setting of protein MS, and it was used to identify the viral protein (Fig. 1b). The protein sample of the health rice was set to negative control.

Plant material and virus sources

Rice plants infected with SRBSDV or RRSV were collected from Yunnan Province, Guizhou province, Guangxi Autonomous Region, Chongqing City, Hunan Province, China from 2011 to 2013. All rice samples had been stored at -80°C until be used. WBPH and BPH were grown in rice infected by SRBSDV or RRSV in plant incubator.

Extraction and determination of protein sample

The total proteins of rice plant were extracted using a modified trichloroacetic acid/acetone procedure as described previously [32]. After adding 1 % Polyvinylpyrrolidone (PVPP), 2 g fresh rice tissue was ground to fine powder at the protection of liquid nitrogen. The fine powder was added into the solution of 10 % trichloroacetic acid (TCA)–acetone (2 % β -mercaptoethanol), and stored at -80°C for overnight. The solution was centrifuged at $8,000\times g$ at 4°C for 30 min, then the precipitate was collected, and added into 10 mL precooling acetone to wash TCA for two times. The precipitate was resolved at the protein lysis buffer [9 M urea, 35 mM Tris, 4 % (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1 % (w/v) dithiothreitol (DTT)]. The protein solution was collected using centrifugation at $12,000\times g$ at 4°C for 15 min. The protein content was determined to 20 $\mu\text{g/mL}$ by Bradford methods using Bio-Rad 680 microplate reader.

Protein separation and protein digestion

Protein separation in shotgun assay was conducted as follow: 20 μg proteins of rice sample were separated via 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), then stained by Coomassie brilliant blue (CBB) and decolorized by the destaining solution. The gel at each lane was cut into 8 groups according to standard protein molecular weight (Page Ruler™ Prestained Protein Ladder, #SM0671, Fermentas) from top to down (Fig. S1a), and stored at 5 % acetic acid solution at 4°C .

Fig. 1 The proteomics profile of the detection of southern rice black-streaked dwarf virus (SRBSDV) and rice ragged stunt virus (RRSV). **a, b** Two modes of shotgun and label free combined with MRM to detect rice virus, respectively. In **a** the words of “higher abundance” and “lower abundance” represent the some viral protein with different expression level; The words of “ingel digest” represent the partial protein in gel was digested by trypsin. In **b** the words of “total protein” represent the protein was extracted from the

viruliferous rice plant; the word of “digest” represent the purified total protein was digested using trypsin; the letter of “mrm” indicated multi reaction monitoring; the letter of “ m/z ” indicated the ratio of molecular weight of the polypeptides and charge. The letter of “LC-MS/MS” represent the peptides sample was injected by NanoLC-1D™ plus system, and the determination of the mass spectrometry was conducted in the mode of first order mass spectrum (MS) and second order mass spectrum (MS²)

CCB were washed from the cut gel using 50 % acetonitrile (ACN) (Merck KGaA, Darmstadt Germany)/ 100 mM NH₄HCO₃ (pH8.0) for 3 times (10 min/per time) at room temperature. The gel were vacuumed by speed vacuum concentrator, then incubated at the solution of 10 mM DTT (Solarbio Co. Ltd, Beijing, PR China)/ 50 mM NH₄HCO₃ (pH8.0) for 1 h at 56 °C. At last, the solution was discarded, and the gel bands were also incubated at 55 mM iodoacetamide (Sigma-Aldrich)/ 50 mM NH₄HCO₃ (pH8.0) for 30 min at dark condition for alkylation. The solution was then discarded, and the gel bands were alternately washed with 10 mM NH₄HCO₃ and 100 % ACN for two times for 10 min for each times, respectively. The gel bands were vacuumed, and digested by trypsin (Sequencing Grade Modified Trypsin, Promega) in 10 mM NH₄HCO₃ at 37 °C for 3 h or overnight. An equal volume of 60 % ACN/5 % formic acid (FA) (Merck KGaA) solution was added the trypsin solution. Polypeptides were released from gel bands by ultrasound during 10 min for two times. The solution was vacuumed, and the precipitate was dissolved in 30 µL H₂O (HPLC grade) containing 0.1 % FA. The supernatant was acquired using the centrifugation at 20 000×g for

30 min at 4 °C for getting rid of impurity and the insoluble substance.

Protein separation in label free assay was conducted as follow: 2 µg proteins of rice sample were digested with trypsin, and the crude polypeptides were filtrated with Millipore tube at the centrifugation of 5,000×g for 10 min, the polypeptides solution was detected using NanoLC-1D™ plus system (Eksigent, Dublin, CA) combined with TripleTOF 5600 MS (Foster City, CA, USA).

Protein identification using protein MS

Separation of protein digests was conducted on NanoLC-1D™ plus system in serial column mode. Firstly, 10 µL (2 µg) of crude polypeptides of injection was performed using a full loop injection, crude polypeptides was desalted on a ChromXP Trap column (Nano LC TRAP Column, 3 µm C18-CL, 120 Å, 350 µm × 0.5 mm, Foster City, CA, USA), then eluted onto a second analytical column—NanoLC C₁₈ reversed-phase column (3C18-CL, 75 µm × 15 cm, Foster City, CA, USA) using a linear gradient formed by A mobile phase (5 % ACN, 0.1 % FA) and B mobile phase (95 % ACN, 0.1 % FA) over 120 min at a

flow rate of 300 nL/min (Table S1–2). TripleTOF 5600 MS was operated in the data-dependent mode to automatically switch between TOF–MS and Product Ion acquisition using Analyst^R Software (TF1.6). The source gas parameters about MS were set by description in support information. β -Galactosidase Digest was used to calibrate as every two samples by 10 min of eluting and 30 min of identifying (Table S1–2).

The analysis of all viral proteins using label free combined MRM technology

At the same condition of source gas, MRM technology was conducted to analyze P2, P3, P5-1, P6, P7-1, P9-1 and P10 of SRBSDV and P6, P3, P8, P9 and NS10 of RRSV. The program of TOF MS (+) was set by description in support information. The information of the precursor ions and the product ion in MRM was set according to the results of Skyline software (version 1.4.0.4421, download from <http://www.absciex.com/downloads>) (Figs. S2, 3). The results come from by MS were extracted using XIC methods via peak view software, and the data was also retrieved by ProteinPilot software. Skyline software were used to analyze the sites of cleavage using trypsin and predicted m/z by as follow [19]. In the window of “Digestion”, “Trypsin [KR/P]” was selected as “Enzyme”, “Max missed cleavages” was set to “0”, “Background proteome” was set to “None”; in the window of “Transition Settings”, “Precursor charge”, “Ion charges” and “Ion types” were set to “2”, “1” and “y”, respectively. “From and to” of Product ions was set to “ $m/z > \text{precursor}$ ” and “3 ions”. The highlighted text in yellow under the brackets represented the digested polypeptides sequence in primary protein structure, the superscript of “+” and “++” of the m/z represented 1 or 2 charges (Figs. S2, 3).

The protein expression abundance was calculated via measuring the peak area of unique polypeptides as follow: the file of “wiff” was open as a “standard TIC” using PeakView software (version 1.2.0.3), the methods of “Dialog (XIC)” was used extracted Ions at +TOF MS (350–1800). Top 20 of Prec MW in ProteinPilot was input in “Specify XIC Ranges”, and width was set 0.05, then the peak area of each precise MW was calculated using “Graph selection information”.

The comparison of sensitivity about mass spectrometry, indirect-ELISA and DIBA

SRBSDV P9-1 was used to verify the sensitivity about the assays of quantitative mass spectrometry, indirect-ELISA and DIBA. SRBSDV genome was extracted using RNA isolation reagent (Takara Co. Ltd., Dalian, China). PCR product of SRBSDV P9-1 was digested with the

endonuclease of *Nde* I and *Eco*R1, and linked to *PET28a* plasmid using T4 DNA ligase. The recombinant plasmid was introduced into the *Escherichia coli* of *DH5a*. SRBSDV P9-1 was purified using Akata purifier 10 (GE Healthcare). SRBSDV P9-1 was separated and identified using SDS-PAGE and protein MS identification. Bovin serum albumin (BSA), ovalbumin (OVA) and SRBSDV P9-1 with different composition ($\mu\text{g}/2 \mu\text{g}$) were prepared in Table S3.

All component was conducted the assays of quantitative mass spectrometry, indirect-ELISA and DIBA. The quantitative mass spectrometry was completed using label free described above. All components were digested by trypsin, and the crude peptides were identified using TripleTOF 5600. The indirect-ELISA and DIBA was conducted previous described [1]. The polyclonal antibodies (1–3) against SRBSDV P9-1 were prepared with polypeptide being as antigen, the information of polypeptide sequence was “CRNDQPTRNTNLSLSQSTENR [20–39]”, “CKDQKDDQSRPTSTDSTKNE [140–159]” and “CKRYRRFRTRIVGNADSVIKSD [319–339]” [1]. The color reaction was conducted using diethanol amine buffer contained with 4-Nitrophenyl phosphate disodium salt hexahydrate (PNPP) and the OD value was determined at 405 nm.

RNA extraction, determination and RT-PCR

Total RNA of rice tissue was extracted using the RNA isolation reagent (Takara Dalian, China) as the protocol. The total RNA was resolved at diethylpyrocarbonate (DEPC) H_2O free RNase, and determined at $\text{OD}_{260/280}$ nm. The sequences of both *S10* (Accession: NC014713) of SRBSDV and *S8* (Accession: NC003758) of RRSV were downloaded from NCBI and the primers was designed using Beacon Designer software. The sequences of the left and right primers of the above genes were 5'-ctccgctgacggttagaag-3' and 5'-ggtcgtaaccgccatagtgt-3', 5'-tgtcgcaattcacaccacg-3' and 5'-gcaatgctaagccgtctcac-3', respectively. The predicted PCR product about these genes were 242 and 395 bp, respectively. All primers of page grade were synthesized by Takara Co. Ltd. For single gene from SRBSDV or RRSV, one step RT-PCR reaction system (Prime Script[®] One Step RT-PCR Kit Ver.2, Takara Co. Ltd., Dalian, China) contained 1 μL of Primer Script Enzyme Mix, 12.5 μL of 2 $\times$ one step buffer, 1 μL of 10 μM of sense primer and antisense primer, 1 μL of RNA sample as template, and 8.5 μL of RNase free water. Reverse transcription was performed at 50 $^{\circ}\text{C}$ for 30 min, then heat denaturation at 94 $^{\circ}\text{C}$. The PCR was performed using the following parameters: 30 cycles at 94 $^{\circ}\text{C}$ for 30 s, 58 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 50 s, followed by one cycle at 72 $^{\circ}\text{C}$ for 10 min. For duplex RT-PCR, the standard RT-PCR were performed using the One Step RT-PCR Kit by

Fig. 2 The expression abundance of all protein coded by the genome of southern rice black-streaked dwarf virus (SRBSDV) and rice ragged stunt virus (RRSV). **a**, **b** in figure represent the expression abundance about all proteins coded by SRBSDV (**a**) and RRSV (**b**)

utilizing 1 μ L (500 ng) of the RNA sample and 1 μ L of 10 μ M of each primer, and a total reaction volume was set at 25 μ L. The cycling conditions were conducted described above. The gene amplification was repeated three times for each sample. Partial PCR product were sequenced using ABI 310 Genetic Analyzer.

Results

The analysis of SRBSDV and RRSV using shotgun proteomics technology

As each separated protein sample in shotgun assay, the proteins were detected from the pattern of differential gels using separation of SDS-PAGE (Fig. S1a). Except for 3 proteins of P5-2, P7-2 and P9-2, others proteins coded by SRBSDV genome had been detected from some gel groups from the sample of viruliferous-rice (Table S4–6). Meanwhile, except for five proteins of P4b, P5, P6, P8a and P8b, others proteins coded by RRSV genome had been detected from some gel groups from the sample of viruliferous-rice (Table S4–6). The identified polypeptides were displayed using protein pilot software and the molecular weights (MWs) and charges of all product ions were indicated “MW” and “Y^{number}” in the spectrum of MS/MS, such as some polypeptides about P9-1 of SRBSDV and P3 of RRSV in Fig. S4, part1F, part2C and others polypeptides of other protein of SRBSDV or RRSV in Fig. S4. Based on the results of un-detected proteins in shotgun assay, the gathering methods, which many bands at the same location in SDS-PAGE was collected at the same sample tube, was used to enhance the concentration of target protein (Fig. 1a). In addition, another mass spectrometer of 4500 MS (Foster City, CA, USA), which being higher than TripleTOF 5600 MS at sensitivity of 100 times, was also

used to detected, but these protein of lower abundance was not detected from SDS-PAGE.

The expression abundance analysis using the approach of MRM combined with label free

As a whole protein sample as an injection into MS, 2 μ g total protein sample was digested without separation via SDS-PAGE, and the crude peptides was injected NanoLC-1DTM plus system. P1, P2, P3, P4, P5-1, P6, P7-1, P8, P9-1 and P10 coded by SRBSDV genome were detected using full scan mode (100–1,800) at the method of protein MS. Meanwhile, at the same experimental condition, P1, P2, P3, P4a, P6, NS7, P8, P9 and NS10 coded by RRSV genome were also detected. P2, P5-1, P4, P8, P7-1, P6 and P10 of SRBSDV has a higher expression abundance, and P3, P4a, P6, P9, NS7, NS10, P8, P1 and P2 of RRSV has also relatively higher protein expression level, comparing with P5-2, P7-2 and P9-2 of SRBSDV, P4b and P5 of RRSV. Nevertheless, P5-2, P7-2 and P9-2 of SRBSDV and P4b and P5 of RRSV rarely expressed at viruliferous-rice (Fig. 2).

The detection of SRBSDV P9-1 and RRSV P3 using MRM approach

Because SRBSDV P9-1 and RRSV P3 were proteins with higher abundance in viruliferous rice sample, the data of the precursor ions about above proteins was introduced into the methods of MRM (Figs. S2, 3), thus the MS was programmed to analyze a preselected group of polypeptides/ions from crud polypeptides [19]. Firstly, SRBSDV P9-1 and RRSV P3 were analyzed using MRM approach against the total crude polypeptides for an injection (2 μ g/10 μ L). The information about the peptide sequence and its *m/z* was calculated using Skyline software (Figs. S2, 3). 4 precursor

ions of SRBSDV P9-1 and 5 precursor ions of RRSV P3 were frequently detected from many viruliferous rice samples infected by SRBSDV or RRSV under the condition of greenhouse and field condition (Figs. S5–13). For instance, the peptide of m/z 599.3⁺⁺ was detected in Fig. S5a, and their product ions (m/z 821.49, 708.40 and 637.37) were also detected using the methods of “Show XIC” and “Show MS and MS/MS” via PeakView software. The m/z data about the precursor ion and their product ions in Fig. S5ab were accordance with the m/z data of the precursor ion (m/z 599.3168⁺⁺) and their product ions (m/z 821.4880⁺, 708.4039⁺ and 637.3668⁺) (Fig. S2b), and the sequence of the peptide of m/z 599.3168⁺⁺ was “R.DFNISLYVRE [83, 92]” (Fig. S2a). Meanwhile, the precursor ion of m/z 716.4⁺⁺ and their product ion (m/z 925.57⁺, 838.54⁺, 725.46⁺ and 612.38⁺) was detected in Fig. S6ab, and the detected information using protein MS was accordance with the information of the precursor ion of m/z 716.4051⁺⁺ and their product ions calculated by Skyline software (Fig. S2b), and the information of polypeptides about the precursor ion of m/z 716.4051⁺⁺ was indicated as “K.FVEMSLPLLR.E [163, 174]” (Fig. S2a); the precursor ion of m/z 1187.6255 and their product ion (m/z 1,469.8414⁺, 1,340.7076⁺ and 1,227.6872⁺) were detected (Fig. S7ab), and the detected information using MS and MS/MS was accordance with the information of the precursor ion of m/z 1,187.5972⁺⁺ and their product ions calculated by Skyline software (Fig. S2ab), and the information of polypeptides about the polypeptides of m/z 1,187.5972⁺⁺ was indicated as “R.ESEESLTEEILEGEGA” (Fig. S2ab); the precursor ion of m/z 896.5058 and their product ion (m/z 1,116.6340⁺, 1,003.5455⁺, 916.5042⁺ and 815.4623⁺) was detected in Fig. S8ab, and the detected information using MS was accordance with the information of the precursor ion of m/z 896.4962⁺⁺ and their product ions calculated by Skyline software (Fig. 2ab), the information of polypeptides of the precursor ion of m/z 896.4962⁺⁺ was indicated as “R.FQLSSLISTPTSILR.I [265, 280]” (Fig. S2ab).

In additional, the precursor ion of m/z 629.8721⁺⁺ and their product ion of m/z 1,046.5849⁺, 878.4964⁺, 731.4243⁺ and 630.3721⁺ about RRSV P3 were detected in Fig. S9ab, the information about +TOF MS and +TOF MS/MS was similarity with the calculated results from Skyline software, and the sequence of the polypeptides about the precursor ion was shown as “K.IVPAFTLEQIK.I [128,138]” (Fig. S3ab). The precursor ions of m/z 471.7850⁺, 891.4578⁺, 779.3809⁺ and 1140.1195⁺ against 4 polypeptides of RRSV P3 were also verified using MRM methods under the condition of +TOF MS and +TOF MS/MS (Figs. S10–13), and these results were agreement with that of Skyline software (Fig. S3ab). The information of polypeptides sequence about four precursor ions were known as “K.EAKPQTLR.F

[81,88]”, “K.ITNDVLSSAQSYLSR.I [139,154]”, “K.LT SQSAADYAFLDR.S [215,228]” and “R.YVENELTIAPT PQDPPIIR.G [871,890]” (Fig. S3a), respectively.

The analysis of rice sample of co-infection about SRBSDV and RRSV using label free combined with MRM

The rice samples of the infection about SRBSDV and/or RRSV at the condition of greenhouse and field were detected using one injection using proteomics technology, some proteins coded by SRBSDV genomes and RRSV genomes was simultaneously detected from viruliferous-rice, such as P6, P10, P9-1, P2, P5-1, P1, P6 and P8 of SRBSDV and P3, P9, P10, P6, P1, P8 and P2 of RRSV (Table S7). The value of “N” indicated the rank of the specified protein relative to all other proteins in the list of detected proteins, top five of SRBSDV about “N” were P6, P10 (major capsid protein), P9-1, P2 and P5-1, respectively; top five of RRSV about “N” were P3, spike protein, P10, P6, P1, respectively (Table S7). The value of “Unused” indicated that a measure of the protein confidence for a detected two viral proteins, and it was calculated from the peptide confidence for peptides from spectra, and the values of “% Cov (Coverage)” indicated that the percentage of matching amino acids from identified peptides had confidence greater than 0 divided by the total number of amino acids in the sequence, the values of “% Cov” about detected protein in Table S7 were over 60.

The comparison of sensitivity about mass spectrometry, indirect-ELISA and DIBA

The products of pET28a-P9-1 and P9-1 gene after digestion by restriction enzyme were separated using agarose gel electrophoresis (Fig. S1b) and the expressed proteins of P9-1 was separated with SDS-PAGE, the molecular mass of target protein was tested by migration at approximately 39.8 kDa (Fig. S1c), and identified with protein MS using standard protocol of protein identification. The protein mixture about P9-1, BSA and OVA was digested, and identified using protein MS. With decreased concentration, 0.03125 µg protein mixture contained with P9-1 still be identified. Nevertheless, the results of indirect-ELISA indicated that the titer of 3 polyclonal antibodies (antibody 1–3) were 0.125, 0.125 and 0.0625 µg, respectively (Fig. 3); the sensitivity of DIBA about 3 antibodies can reached at 0.25 µg, but the assay appeared light dot at the content of 0.125 µg (Fig. 4). So, we conclude that the sensitivity of mass spectrometry was higher than that of indirect-ELISA and DIBA at the condition of titer of polyclonal antibody prepared by our research group.

Fig. 3 The analysis of the titers of the polyclonal antibodies of southern rice black-streaked dwarf virus (SRBSDV) P9-1. Antibody 1–3 in figure represent 3 kinds of polyclonal antibodies prepared via 3 kinds of polypeptide antigen. The value in figure represent the value of absorbance value (mean $\pm$ standard deviation) at condition of the different concentration of SRBSDV P9-1 from 2 to 0 μ g. The graph was prepared with the software of Origin 8.0

Fig. 4 The analysis of DIBA of southern rice black-streaked dwarf virus (SRBSDV) P9-1 antibodies at condition of the different concentration of SRBSDV P9-1. The number of “1–9” represent the concentration of SRBSDV P9-1 from 2 to 0 μ g

Verification the results of protein MS using simplex RT-PCR and duplex RT-PCR

The rice seeding was transmitted using viruliferous WPBH or BPH under the condition of greenhouse, and specific RT-PCR assays were conducted via the two positive samples. The predicted PCR products were 242 (SRBSDV) and 395 bp (RRSV), respectively (Fig. S14a–c). The established method can simultaneously detect the two viruses in infected rice by distinguishing the size of the amplified products, then was used to detect the rice sample from many areas in China (Fig. S14d), and the results about single infection or co-infection was shown in Table 1. The results of PCR about the rice sample, which was collected from many areas and infected under the condition of greenhouse, was agreement with the results of the protein MS. For instance, the rice sample from Jiucheng indicated

the phenomenon of co-infection about two virus, other rice sample collected by our research group indicated single infection of SRBSDV (Table 1).

Discussion

So far, MS techniques and proteomics technology were known as a burgeoning technology to be used to detect plant virus [21]. MRM (also referred to selected reaction monitoring, SRM) are developed to detect fragment-ion signals arising from unique diagnostic peptides representing each of the viral targeted proteins [2]. In this study, shotgun technology combined with full scan mode (100–1,800) was used to identify the kinds of protein coded by two virus's genome (Table S4). P2, P5-1, P6, P7-1, P9-1 and P10 coded by SRBSDV genome were identified from many bands in SDS-PAGE, and P2, P3, NS7, P8 and NS10 coded by RRSV genome were also identified, but some proteins wasn't detected from all bands even enhancing loading sample or collecting many sample from same location at SDS-PAGE (the mode of Fig. 1a), these results indicated that these proteins may be differential function in plant host or at different stage of infection. So, label free technology was used to study the expression abundance about all protein coded by viral genome via LC method of one injection of 2 μ g loading sample combined with MRM at full scan mode (Fig. 2), the result was also indicated the trends of up- or down-regulation of proteins coded by SRBSDV and RRSV was accordance with that of shotgun assay. Some structural protein and nonstructural protein related with viral replication or viral formation had a higher amount in rice plant (Fig. 2). These results were basically similarity with the results of real-time PCR, and *S1*, *S2*, *S3*, *S5-1*, *S7-1*, *S8*, *S9-1* and *S10* of SRBSDV had also an up-regulation trends at the levels of RNA in rice plant [5]. For instance, a higher abundance about P9-1 of SRBSDV in rice plant was verified to P9-1 as a significant protein involved with viroplasms formation [12]. In additional, gene of *S7-2* and *S9-2* of SRBSDV had also an up-regulation trends in rice plant at RNA level [5], and *S5-2* gene of SRBSDV expressed had an expression level at a certain extent, with level of *S5-2* gene being higher than that of *S10* gene [5], these results were seemingly contradiction with our results of un-detection of P5-2, P7-2 and P9-2 and up-regulation of P10 in rice plant (Table S4; Fig. 2). In additional, our results were similarity with the results of P7-1 and P7-2 of rice black streaked dwarf virus (RBSDV), P7-1 had a higher expression level in rice plant and P7-2 rarely expressed in rice plant [10, 18]. These results indicated that the mechanism of gene expression was complexity, which existed many steps of regulation. Some reports indicated that the length of ORF in upstream and the flanking sequence of initial codon of AUG in upstream can induce the expression

Table 1 The detection of rice samples from some provinces in China using the assays of one-step duplex RT-PCR and protein MS

Sample collection time	Sample origin	Sample number	Positive sample number				Co-infection sample number	
			SRBSDV		RRSV		PCR	Protein MS
			PCR	Protein MS	PCR	Protein MS		
2012	Jiucheng, Shidian County, Yunnan Province	23	14	14	17	17	8	8
2013	Jiucheng, Shidian County, Yunnan Province	45	28	28	31	17	14	14
2012	Chaoyang, Libo County, Guizhou Province	18	18	0	0	0	0	0
2013	Chaoyang, Libo County, Guizhou Province	5	5	0	0	0	0	0
2012	Chengxi, Bobai County, Guangxi	7	7	0	0	0	0	0
2012	Lirang, Liangping County, Chongqing	4	4	0	0	0	0	0
2012	Baimangying, Jianghua County, Hunan Province	12	12	0	0	0	0	0

level of 2 ORF of RBSDV [18]. P3, P4a, NS6, NS7, P9 and NS10 of RRSV had a higher expression level at rice plant (Fig. 2). For instance, the phenomenon of P3 of RRSV with higher expression level in rice plant was accordance with the results of P3 serving as capsid shell protein [4]. P4b of RRSV wasn't detected from rice plant via many methods in our study, the results indicated that P4b may be not a significant protein to the replication of RRSV, this results was also similarity with Upadhyaya's study [25].

Based on the rice sample of co-infection about SRBSDV and RRSV, the interaction mechanism between viruses has been not reported. We indeed detect many proteins of SRBSDV and RRSV from rice samples of co-infection using MRM technology combined with label free (Table S7, Fig. 2), the sensitivity about mass spectrometry, indirect-ELISA and DIBA was also compared in this study, the sensitivity of mass spectrometry was higher than that of indirect-ELISA and DIBA. So we can conclude that P5-2, P7-2 and P9-2 of SRBSDV and P4b and P5 of RRSV may be lower expression level in rice plant, and these proteins may be not as markers of protein in using diagnostic, and SRBSDV P9-1 and RRSV P3 can be used to be molecule marker in proteomics assays.

Some proteins of SRBSDV and RRSV were detected from infected rice sample using shotgun proteomics technology, and determined using MRM method combined with label free. Some structural proteins and non-structural proteins were found to higher expression abundance, and P5-2, P7-2 and P9-2 of SRBSDV and P4b and P5 of RRSV has a lower expression abundance. For two proteins with higher abundance expression, SRBSDV P9-1 and RRSV P3 were selected to protein marker to develop the detection methods in mass spectrum of MRM.

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