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Comparative proteome analysis of rubber latex serum from pathogenic fungi tolerant and susceptible rubber tree (*Hevea brasiliensis*)

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ABSTRACT

Many cultivated rubber trees (*Hevea brasiliensis*) are invaded by various *Phytophthora* species fungi, especially in tropical regions which result in crop yield losses. Comparative proteome analysis coupled with liquid chromatography electrospray/ionization (LC-ESI) mass spectrometry identification was employed to investigate the relative abundance of defense related proteins in *Phytophthora* sp. susceptible (RRIM600) and tolerant (BPM24) clones of rubber tree. Proteome maps of non-rubber constituent of these two model clones show similar protein counts, although some proteins show significant alterations in their abundance. Most of the differentially abundant proteins found in the serum of BPM24 illustrate the accumulation of defense related proteins that participate in plant defense mechanisms such as beta-1,3-glucanase, chitinase, and lectin. SDS-PAGE and 2-D Western blot analysis showed greater level of accumulation of beta-1,3-glucanase and chitinase in latex serum of BPM24 when compared to RRIM600. A functional study of these two enzymes showed that BPM24 serum had greater beta-1,3-glucanase and chitinase activities than that of RRIM600. These up-regulated proteins are constitutively expressed and would serve to protect the rubber tree BPM24 from any fungal invader. The information obtained from this work is valuable for understanding of defense mechanisms and plantation improvement of *H. brasiliensis*.
Biological Significance: Non-rubber constituents (latex serum) have almost no value and are treated as waste in the rubber agricultural industry. However, the serum of natural rubber latex contains biochemical substances. The comparative proteomics analysis of latex serum between tolerant and susceptible clones reveals that the tolerant BPM24 clone contained a high abundance of several classes of fungal pathogen-responsive proteins, such as glucanase and chitinase. Moreover, other proteins identified highlighted the accumulation of defensive-associated proteins participating in plant fungal immunity. The isolation of beta-1,3-glucanase, chitinase, and lectin from latex serum should be further investigated and may provide a therapeutic application. This investigation will lead to possible use of latex serum as a great biotechnological resource due to the large quantity of serum produced and the biochemicals contained therein.

1. Introduction

The rubber tree (*Hevea brasiliensis*) is a well-known industrial crop cultivated widely in Southeast Asia. The latex of the rubber tree is composed of two major components, rubber and non-rubber (called serum). The rubber latex can be collected simply by engraving or tapping the bark of the tree. Rubber constituents contain polyisoprene which is an economically important natural polymer. However, non-rubber constituents have almost no value and are treated as waste in the rubber agricultural industry. The serum of natural rubber latex contains biochemical substances which include proteins and peptides. Several defense associated proteins against fungal phytopathogens were identified to be present in the non-rubber constituents of natural rubber latex, for instance hevein [1], chitinase [2], early nodule-specific protein homolog [3], protease inhibitor [4], cystatin [5], and AP2/ERF transcription factors [6]. In our study, *H. brasiliensis* BPM24 and RRIM600 were selected to be representative of fungal tolerant and susceptible clones, respectively. Varieties of rubber tree considered as high yielding clones are RRIT251, RRIT226, RRIM600, and BPM24. The RRIM600 is the most productive *Hevea* clone which is extensively planted in the South of Thailand. This area has high humidity which exposes the rubber trees to infection from several pathogenic fungi including various *Phytophthora* species [7] that cause leaf fall and black stripe diseases [8]. The RRIM600 is susceptible to these diseases which results in heavily reduced production of natural rubber latex [9]. The BPM24 also produces a high yield of latex, is highly tolerant to *Phytophthora* disease and moderately tolerant to black stripe, bird eye spot and pink diseases.

A study of the relative abundance of defense related proteins is necessary to increase our understanding of the phytopathogen tolerant

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mechanisms in plants, especially in an important industrial crop like the rubber tree. The information obtained may also be valuable for improvement of *H. brasiliensis*. In the present study we used two-dimensional gel electrophoresis followed by LC–MS/MS to identify constitutively abundant proteins that may be involved in tolerance to *Phytophthora* species. For this study we used the latex serum from tolerant BPM24 and compared it to the latex serum from susceptible RRIM600. We identified several defense related proteins that were differential abundance in the latex serum of these two *Hevea* clones.

2. Materials and methods

2.1. Sample collection

Fresh latex samples were obtained from the rubber trees, *H. brasiliensis*, clones RRIM600 and BPM24 that were approximately 10 years old and planted in the same crop in Huai-Yot district, Trang province in the southern part of Thailand. All of these rubber trees were *Phytophthora*-free plants (non-infected plants). The rubber trees were normally tapped for fresh latex using Jeh-Bong knife. Each group contains six rubber trees. The first 5–10 drops of latex were discarded to avoid contaminants. Fresh latex was collected directly into tubes containing lysis solution (final concentration 8 M Urea, 2 M Thiourea, 2% CHAPS, 0.4% Triton X-100, 50 mM DTT, 1X Protease inhibitor cocktail [Roche], 1X PhosStop [Roche]). The solution was thoroughly mixed until the latex was completely solubilized then placed in an ice bath and immediately taken back to the laboratory.

2.2. Latex serum isolation, protein extraction and precipitation

The non-rubber constituents of each sample were collected after centrifugation at 12,000 ×*g* for 30 min at 4 °C. The isolated latex layer was resuspended with lysis solution 2–3 times to increase the protein extraction yield. The extracts were centrifuged 2–3 times to remove solid contaminants. Then methanol–acetone was used to precipitate proteins in the extracts. Briefly, the extracted solutions were incubated with 2 volumes of cold acetone and methanol (4:1) and incubated at −20 °C for overnight. The precipitated proteins were pelleted by centrifugation at 16,000 ×*g* for 15 min at 4 °C and washed three times with cold acetone. The pellets were allowed to air-dry at room temperature. The pelleted proteins from this non-rubber serum were solubilized in lysis solution. Protein concentration was determined by Bradford's assay using bovine serum albumin (BSA) as the standard.

2.3. Protein gel electrophoresis (1-D PAGE and 2-D PAGE) and Western blot analysis

For 1-D PAGE, 15 μg of proteins from BPM24 and RRIM600 were resolved by 12.5% SDS-PAGE with a constant voltage of 120 V for 2.5 h. The resolved gels were stained by colloidal Coomassie blue G-250 containing 10% ammonium sulfate, 2% phosphoric acid and 0.001% of Brilliant Blue G [USB] to observe protein profiling of each clone. For 2-D PAGE, 200 μg of extracted proteins obtained from an individual rubber tree was loaded onto an immobilized pH gradient (IPG) strip (7 cm, nonlinear pH 3–10; GE Healthcare). Six IPG strips for each group were analyzed. Each strip was passively rehydrated overnight with 200 μg of total proteins that were premixed with a rehydration solution containing 7 M Urea, 2% CHAPS, 2% ampholytes (pH 3–10), 120 mM DTT, 40 mM Tris-base and bromophenol blue. The first dimension of 2-DE was carried out in IPGphor IEF system [GE Healthcare, Sweden]. The isoelectric focusing was performed at 50 mA per strip at 20 °C using a continuous increase in voltage (up to 5000 V) to reach 12,100 V h. The focused IPG strips were incubated in an equilibration buffer containing 30% glycerol, 20% sucrose, 2% SDS, 50 mM Tris–HCl (pH 8.8), 100 mM DTT, and 0.002% bromophenol blue. The second dimension was conducted on miniVE Vertical Electrophoresis System [GE Healthcare]. The equilibrated IPG strips were placed onto the top of a 12.5% SDS-PAGE gel and the samples resolved with a constant voltage of 120 V for 2.5 h. The resolved proteins were transferred to a nitrocellulose membrane [GE Healthcare] using a Mini Trans-Blot electrophoresis transfer cell [Bio-Rad, CA]. The membranes were presoaked and equilibrated in Tris–glycine transfer buffer containing 39 mM glycine, 0.04% SDS, 10% methanol, and 48 mM Tris–HCl; pH 8.3. Unstained membranes were prepared for hybridization by incubation with blocking solution [5% BSA in TBS buffer (20 mM Tris–HCl, and 150 mM NaCl; pH 7.4) containing 0.1% (*v*/*v*) Tween 20] at 4 °C overnight to prevent nonspecific binding. The membranes were incubated with rabbit polyclonal anti-beta-1,3-glucanase [Agrisera AB] with a dilution of 1:10,000 or rabbit polyclonal anti-chitinase [Agrisera AB] in 2.5% skim milk in 0.1% TBS-tween for 3 h at room temperature. After washing with 0.1% TBS-tween, the membrane was incubated with goat-anti rabbit secondary antibody conjugated with horseradish peroxidase [Santa Cruz Biotechnology] at a dilution of 1:15,000 at room temperature for 1 h. After three washes with 0.1% TBS-tween, immunoreactive spots were visualized with the SuperSignal West Pico Chemiluminescent Substrate [Pierce, IL]. Gels and blots were scanned by using an Image Scanner II [GE Healthcare].

2.4. Image analysis, in-gel digestion and mass spectrometry

Comparative analysis of protein spots from BPM24 and RRIM600 was performed by analysis with Image Master 2D Platinum software version 7.0 [GeneBio, Switzerland]. For matching and quantitative intensity analysis, the reference gel was then used for matching the corresponding protein spots among 2-D gels. The matched protein spots by an automatic matching were then confirmed and edited manually. Matching of protein spots across different gels must be consistently found in all triplicated gels. Background subtraction was performed, and the intensity volume of each spot was normalized with total intensity volume (summation of the intensity volumes obtained from all spots within the same 2-D gel). After analyzed spot matching, the protein spots with significant changes in intensity volume (Student's T-test; $p < 0.05$) and with a ratio of normalized volume intensity, BPM24 compared to RRIM600, which was more than 2-fold different were excised with a sterile scalpel for in-gel digestion. Briefly, the gel pieces (1 mm^2) were washed with 50% acetonitrile in 25 mM ammonium bicarbonate (pH 8.5) for 15 min twice to remove Coomassie dye. After dehydration with 100% acetonitrile for 5 min at room temperature, the gel pieces were rehydrated with Trypsin Gold [Promega], Mass Spectrometry Grade (10 ng/μl trypsin in 25 mM ammonium bicarbonate) at 37 °C overnight. The resulting peptides were then subjected to nanoliquid chromatography coupled with electrospray ionization tandem mass spectrometry (nanoLC-ESI–MS/MS). The MS/MS data were output as a searchable *.pkl* or *.mgf* file. The resulting file was then searched with the Mascot search engine [Matrix Science, MA] using the NCBInr database (NCBInr 20140107). Parameters for the MASCOT search were peptide mass tolerance of 1 kDa; MS/MS ion mass tolerance of 1 Da; maximally one missed cleavage; and tryptic digestion. Only matched proteins with significance scores ($p < 0.05$) were reported.

2.5. Multiplexed gel staining

Two dimensional electrophoresis experiments were conducted in triplicate to stain phospho- and glycoproteins. After separation, the protein spots were then stained with Pro-Q Diamond Phosphoprotein Gel Stain [Molecular Probes, OR]. Briefly, the gels were fixed in 50% methanol and 10% acetic acid and then washed with 18 MOhm milli Q water. The gels were stained in Pro-Q Diamond phosphoprotein gel stain with gentle agitation in the dark for 90 min. After staining, the gels were destained in 20% (*v*/*v*) acetonitrile and 50 mM sodium acetate, pH 4.0 for 1.5 h at room temperature and protected from light. Subsequently, these gels were washed and stained with SYPRO Ruby protein gel stain [Molecular Probes, Invitrogen] overnight in the dark. The gels

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