

Proteomic analysis of soybean leaves in a compatible and an incompatible interaction with *Phakopsora pachyrhizi*

Abstract

Asian soybean rust (ASR), which is incited by the fungus *Phakopsora pachyrhizi*, is considered one of the most aggressive diseases to the soybean culture. There are no commercial cultivars immune to the pathogen and the control measure currently used is the application of fungicides that harms the environment and increases production costs. For a better understanding of the host's response to the pathogen at the molecular level, two soybean genotypes were analyzed (PI561356, resistant to ASR and Embrapa 48, susceptible) at 72 hours and 192 hours after inoculation with spores of *P. pachyrhizi*. Leaf protein profiles of the plants were compared by two-dimensional electrophoresis associated with mass spectrometry (MS). Twenty-two protein spots presented different levels when the two treatments were compared (inoculated vs. non-inoculated). From those, twelve proteins were identified by MS analysis. Some of them are involved in metabolic pathways related to plant defense against pathogens, as in the case of carbonic anhydrase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, fructose-bisphosphate aldolase and glutamine synthetase. The possible biochemical-physiological meanings of our findings are discussed.

Keywords

Glycine max • *Phakopsora pachyrhizi* • 2-D electrophoresis • MALDI • mass spectrometry

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1. Introduction

Among the main factors limiting soybean (*Glycine max* (L.) Merrill) crop are the diseases [1]. More than 100 diseases can affect this culture [2], of those at least 50 have already been identified in Brazil [3].

Asian soybean rust (ASR), incited by the fungus *Phakopsora pachyrhizi* Sydow, is a major disease limiting soybean production in tropical and subtropical areas worldwide causing yield losses from 10 to 80% [4]. In 2001, ASR became one of the most relevant problems in Brazilian agriculture [5].

P. pachyrhizi infects more than 150 species of plants from more than 53 genera including soybean, related *Glycine* species, and other hosts in the Fabaceae [6]. It is an obligate biotrophic plant-pathogenic fungus that colonizes leaf tissue [7], causing rapid yellowing and premature leaf fall, hampering full grain formation [8,9]. The life cycle begins with uredospores coming uredinias produced into infected plants in other soybean fields. Under favorable conditions, after reaching the leaves, the spores germinate with a minimum of six hours, and the symptoms may appear within five to seven days. The urediniosporal lesions begin to be produced from nine to twelve days after germination and

penetration [10]. Symptoms begin in the lower leaves of the plant and are characterized by tiny dots of darker color compared to healthy tissue [9]. In these dark spots, lower protuberances are observed characterizing the beginning of the formation of fruiting structures of the fungus. Progressively, the bulge acquires color light brown to brown-black, which opens up pores that expel uredospores [11].

At least six ASR resistance genes have been described so far: *Rpp1* [12], *Rpp2* [13], *Rpp3* [14], *Rpp4* [15], *Rpp5* [16] and *Rpp6* [17]. The immune reaction, where no visible symptoms are observed, has only been reported with *Rpp1* when inoculated with certain isolates [4,7]. Resistance responses mediated by the *Rpp2* to *Rpp6* loci limit fungal growth and sporulation through the formation of visible reddish-brown lesions suggestive of a hypersensitive-like response (HR) [16,18]. Tan-colored lesions and fully sporulating uredenia generally indicate a susceptible interaction to ASR [7]. The resistance sources identified are all specific to certain strains of *P. pachyrhizi* [18]. Therefore, the effectiveness of these genes *R* is limited (partial) due to the high virulence and variability of the pathogen [19]. There are no immune commercial cultivars available and the current control measures include the heavy

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use of fungicides which increases production costs and harm the environment [7,20].

However, genetic resistance is the most effective and inexpensive measure for disease control [7]. Understanding the molecular, physiological and cellular mechanisms involved in the soybean response to this pathogen is an excellent strategy to screen genes involved in the activation of metabolic pathways related to the defense of the host to the pathogen. Understanding the host responses at the molecular level is certainly essential for effective control of the disease, and the molecular basis for rust resistance remained largely unknown in soybean [21]. The study of soybean genes that are differently expressed as a response to *P. pachyrhizi* can aid the identification of important genes involved in the resistance response and provide tools for genetic breeding programs for creation of soybean cultivars resistant to ASR [22,23]. Although molecular studies are being conducted on this pathosystem, there is still little information on the molecular basis of this interaction [21,24-26]. To develop rust-resistant soybean cultivars, a better understanding of the molecular basis and essential genes involved in defense responses is a key to control this disastrous disease [21].

The proteome can be defined as the set of proteins present in a tissue, cell or biological system in a given moment of cell life, or as the profile of cell proteins expressed by the genome of an organism under a determined physiological situation. The proteomic analysis refers to the systematic assessment of the proteome, in order to compare the gene expression of cells, tissues or organisms in distinct physiological moments, for example, in sick and healthy tissues, treated and non-treated, resistant and susceptible [27]. Recently, Wang et al. (2012) [21] identified proteins with altered levels in a resistant soybean cultivar (SX6907) at two different time points (12 and 24 hours) upon inoculation with *P. pachyrhizi*. Using the same methodologies like two-dimensional electrophoresis (2-DE) and mass spectrometry (MS), the present study aimed at identifying proteins that accumulate differentially in response to *P. pachyrhizi* in two soybean genotypes, PI561356, carrying the *Rpp1* gene [19] (resistant) and Embrapa 48 (susceptible) at two different time points (72 and 192 hours) after inoculation. As a result of this interaction, twelve differentially accumulated proteins were detected and identified in this study. These proteins participate in metabolic pathways related to plant defense to pathogens.

2. Methods

2.1 Plant material and inoculation

Seeds of the genotypes used in this experiment were provided by the Soybean Active Germplasm Bank of Embrapa Soja, Londrina – PR, Brazil. The plants were sprayed at developmental stage V2 with a suspension of a population of *P. pachyrhizi* collected in the Embrapa Soja fields, located in Londrina, Paraná state, and maintained on cultivar BRMSM-Bacuri in a greenhouse. Urediniospores were collected by

tapping infected leaves over a plastic tray. The spores were then resuspended in distilled water containing 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20) to a final concentration of approximately 70,250 spores/mL. This concentration was defined by preliminary infection experiments performed by our breeding group. The leaves from two distinct genotypes (PI561356, resistant to ASR and Embrapa 48, susceptible) were collected 72 and 192 h.a.i. (hours after inoculation). At 72 h.a.i. soybean response to the pathogen is intense [22-24] and also close to 192 h.a.i. when the fungus reaches the uredinia (urediniospores) production stage [26]. For each genotype and each time point, leaves of control plants (*mock*: non-inoculated) were also collected. Eight treatments were analyzed, with three biological replications for each treatment.

2.2 Protein extraction

Leaf protein extraction was based on the SDS/phenol method [28] with some modifications. The leaves (2-3 g) were powdered in the presence of liquid N₂ in a mortar and pestle. The powder was transferred to a 50 mL Falcon tube. After N₂ evaporation, 20 mL extraction buffer (1% polyvinylpyrrolidone, 2% β-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride (PMSF) in cold acetone) were added to the tube. After brief vortexing, the mixture was sonicated at 30% of the maximum power of the UltraSonic Processor Model GE-50 (*Thomas Scientific*, Swedesboro, New Jersey, USA) (amplitude of 70 dB) and centrifuged at 6,000 g, at 4 °C, for 15 min. The pellet was washed successively in acetone (twice), 10% trichloroacetic acid (TCA) in acetone (four times), 10% TCA in water (twice), 80% acetone (twice) and 80% ethanol (once), and dried overnight at room temperature. For the washes the pellet was resuspended by vortexing and centrifuged at 6,000 g, for 10 min, at 4 °C.

The final pellet obtained was resuspended in 10 mL dense SDS buffer (0.75M sucrose; 2% sodium lauryl sulfate (SDS); 0.1M Tris-HCl, pH 8.0; 2% β-mercaptoethanol; 1mM PMSF) and kept at room temperature for 10 min. The mixture was transferred to a 15 mL Falcon tube to which 5 mL buffered phenol (pH 8.0) were added. The mixture was kept on ice for 10 min. During this period the mixture was vortexed three times for 30s each time and then centrifuged at 6,000 g for 10 min, at 4 °C. The phenolic phase was transferred to a fresh 50 mL Falcon tube to which 20 mL 0.1M ammonium acetate in methanol were added. After 30 min at -20 °C, the tube was centrifuged at 6,000 g for 15 min, at 4 °C. The pellet obtained was washed as previously described in 0.1M ammonium acetate in methanol (twice), 80% acetone (twice) and 70% ethanol (once). The final pellet was dried at room temperature overnight, resuspended in sample buffer (7M urea, 2M thiourea and 4% (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS)) and sonicated at 10% of the maximum power of the UltraSonic Processor Model GE-50 (*Thomas Scientific*, Swedesboro, New Jersey, USA). The Bradford Method [29] was used for protein quantification and the protein extract was stored at -80 °C.

2.3 Separation of total proteins by two-dimensional gel electrophoresis (2-DE)

2.3.1 Rehydration and sample loading

The samples (1,000 µg of proteins) were loaded during the reswelling process in a rehydration apparatus IPG BOX (*GE Healthcare*, Piscataway, NJ, USA) at 20 °C for 12 h. For the first dimension, 24 cm gel strips were used (with a linear pH gradient ranging from 3.0 to 10.0). To rehydrate each strip, 450µL of a mixture containing the solubilized proteins in 7M urea, 2M thiourea, 4% CHAPS and 2.5% IPG buffer plus DeStreak solution (*GE Healthcare*, Piscataway, NJ, USA) [30] were used.

2.3.2 Isoelectric focusing (IEF)

IEF was conducted in the equipment IPGphor III (*GE Healthcare*, Piscataway, NJ, USA). Electrophoresis conditions were according to the *GE Healthcare Handbooks* [30], with some modifications: 1) 200 V for 18 h; 2) 500 V for 1 h; 3) 800 Vh in gradient until 1,000 V; 4) 16,500 Vh in gradient until 10,000 V; 5) 27,000 Vh in one step of 10,000 V. After the IEF, the strips were stored at -80 °C until the second dimensional electrophoresis.

2.3.3 Equilibration of the gel strips

After the IEF, the strips were equilibrated in 10 mL equilibrating buffer (75mM Tris-HCl, pH 8.8, 6M urea, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue) in two 30-min stages in order to reduce and alkylate the proteins. In the first stage, 180 mg dithiothreitol (DTT) were added to the equilibrating buffer. In the second stage, 430 mg iodoacetamide were added to 10 mL fresh equilibrating buffer [30]. The strips were then briefly incubated in 1X running buffer [31] and submitted to the second electrophoresis dimension (SDS-PAGE).

2.3.4 Electrophoresis in polyacrylamide gel (SDS-PAGE)

The second dimension electrophoresis was based on Laemmli (1970) [31] in a 12.5% polyacrylamide gel (30% acrylamide, 2.6% N,N'-methylenebisacrylamide), in a DaltSix unit (*GE Healthcare*, Piscataway, NJ, USA). Separation was performed at 10 mA/gel for 45 min and then at 40 mA/gel until the bromophenol blue reached the gel lower limit. The temperature was kept at 8 °C using a thermostatic circulator.

2.3.5 Analysis of the spots

The 2D gels were stained with coomassie blue G-250 [30]. They were then photo digitalized in an Image Scanner III (*GE Healthcare*, Piscataway, NJ, USA) and the images were calibrated with the aid of the software *Labscan* (*GE Healthcare*, Piscataway, NJ, USA). For the comparative analysis of the images, the software *ImageMaster 2D Platinum 7.5* (*GE Healthcare*, Piscataway, NJ, USA) was used. Image analysis included spot detection, spot measurement, background subtraction and spot matching of three biological replicates gel. Prior to performing spot matching between gel images, one gel image was selected as reference. The amount of protein of each spot was expressed as the

volume of that spot which was defined as sum of the intensities of all the pixels that make up that spot. To correct the variability and to reflect the quantitative variations of protein spots, the spot volumes were normalized as a percentage (%Vol) of the total volume in all the spots in the gel. The ratio method was used for the overlapping analysis between the mock control and the inoculated treatment for each genotype and each inoculation time, and to evaluate gene expression changes. Matched spots showing a ratio overlap values over 1.5 and significant one-way ANOVA ($p < 0.05$) analysis were considered over or under accumulated.

2.4 In-gel protein digestion

The regions containing the gel spots corresponding to the differentially accumulated proteins were removed from the gel and the proteins were submitted to trypsinolysis [32]. The gel pieces were transferred to siliconized PCR tubes (200µL) previously washed with methanol. Destaining was performed in four washes, two of 1 h each, one overnight, and one of 1 h the next day. All the washes were done at room temperature, with agitation (750 rpm) in a thermomixer (*Eppendorf*, Hamburg, Germany) with a 50% acetonitrile solution containing 25mM ammonium bicarbonate, pH 8.0. The destaining solution was discarded and the gel pieces were dehydrated in pure acetonitrile for two 5-min periods and dried in a Speed Vac Concentrator Plus (*Eppendorf*, Hamburg, Germany) for 15 min. The proteins were then reduced with 65 mM DTT in 100 mM ammonium bicarbonate, pH 8.0, for 30 min, at 56 °C, in a thermomixer, at 500 rpm. After this step, the proteins were alkylated with 200mM iodoacetamide in 100mM ammonium bicarbonate, pH 8.0, for 30 min, at room temperature, in the absence of light, in a thermomixer, at 500 rpm. Sequentially, the gel pieces were twice washed in 100 mM ammonium bicarbonate, pH 8.0, for 10 min, dehydrated in pure acetonitrile for 5 min and after an additional dehydration step they were dried in a Speed Vac for 15 min. For the tryptic digestion Trypsin Gold V5280, mass spectrometry grade (*Promega*, Madison, Wisconsin, USA) was used. A stock was prepared out of 100µg enzyme in 100µL 50mM acetic acid (1,000 ng/µL). For the cleavage solution the stock enzyme solution was diluted in 40mM ammonium bicarbonate, pH 8.0, in 10% acetonitrile to a final concentration of 25 ng/µL. To each tube 20µL cleavage solution was added, enough to cover the gel pieces. The tubes were kept on ice for 45 min to keep the enzyme inactive and prevent premature trypsinolysis. After this period, 50µL 40mM ammonium bicarbonate, pH 8.0, in 10% acetonitrile were added to each tube. The samples were then incubated at 37 °C for 16 h in a thermomixer at 500 rpm. After digestion, the gel pieces were incubated in an ultrasonic bath Model USC 1880 (*Thornton-Unique*, Indaiatuba, SP, Brazil) for 10 min, vortexed for 20 s and the solution containing the tryptic peptides was transferred to a fresh tube. The remaining peptides in the gel pieces were removed in two sequential stages by adding to each tube 30µL 5% formic acid in 50% acetonitrile, vortexing for 20 s and incubating 15 min at room temperature, 2 min in the ultrasound bath and additional vortexing for 20 s. The

solution was removed and added to the tube containing the first round extract. The samples, containing tryptic peptides, were concentrated until about 10 μ L in a SpeedVac and later desalted in a Zip Tip C18 column (*Millipore*, Billerica, MA, USA).

2.5 Mass spectrometry and protein identification

Mass spectra of the tryptic peptides were obtained in a MALDI-TOF/TOF mass spectrometer model Ultraflex III (*Bruker Daltonics*, Bremen, Germany). The samples of tryptic peptides were mixed with α -cyano-4-hydroxyl cinnamic acid (*Bruker Daltonics*, Bremen, Germany) in a proportion of 1:1. The mass spectra obtained were processed using Flex analysis software (*Bruker Daltonics*, Bremen, Germany) and a peak list (xml and mgf format) was used for identification of the proteins by the peptide mass fingerprinting (PMF) method and by peptide fragment fingerprinting (PFF), respectively using the Mascot software against the NCBI (<http://www.ncbi.nlm.nih.gov/>), Swissprot (http://web.expasy.org/docs/swiss-prot_guideline.html) and *Glycine max* (Phytozome) (<http://www.phytozome.net/search.php>) protein databases. For the search the following parameters were considered: a mass tolerance of 50 ppm for the parental ion, fixed modification for carbamidomethylation of cysteine residues and variable modification for oxidation of methionine residues. For positive identification, the following criteria were used: significant Mascot score (p-value < 0.05) for at least six peptides showing matches (PMF). Additionally, positive protein assignments required greater than 20% sequence coverage and less than 25% deviation between theoretical and experimental MW values obtained from calibrated 2D gels. Positive identifications by PFF were considered valid for at least three peptides with significant Scaffold score (p>95%) for peptides and proteins, after visual inspection of matches. All identifications were verified manually.

3. Results

At the time point of 72 h.a.i., 716 well resolved spots on average were detected in the six gels loaded with total proteins from the susceptible soybean genotype Embrapa 48 (inoculated and mock plants). The number of matches was 580, but no spot with significant variation in %Vol were detected. In the case of resistant genotype PI561356, 711 well resolved spots were detected, with 552 matches, of which 13 showed significant variation in %Vol and nine were identified using high quality MS spectra (Table 1 and Figure 1A): translationally controlled tumor protein (TCTP), gamma-glutamyl hydrolase, small subunit ribosomal protein S1, elongation factor 1-alpha, three binding proteins to Rubisco subunit beta, glutamine synthetase and fructose-bisphosphate aldolase.

At the time point of 192 h.a.i., 1,449 well resolved spots were detected in the gels corresponding to genotype PI561356 (inoculated and mock control plants), with 790 matches. Four spots with significant variation in %Vol were detected but the quality of the mass spectra of the corresponding proteins did not allow their proper identification. For genotype Embrapa 48, 1,367

well resolved spots were detected, with 779 matches, and five spots showed significant variation in %Vol, three of which were identified (Table 1 and Figure 1B): chloroplast glyceraldehyde-3-phosphate dehydrogenase subunit A (GAPDH α), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) and carbonic anhydrase. The protein accumulation level differences between inoculated and mock plants based on spot %Vol at 72 and 192 h.a.i. are depicted in Table 2.

4. Discussion

Identification of differentially accumulated proteins in both time points analyzed enabled some inferences on their possible roles during host-pathogen interaction. In our analysis, we found a reduced synthesis of TCTP in genotype PI561356 72 h.a.i.. Comparatively, Hill et al. (1999) [33] analyzed genes of *Gossypium hirsutum* expressed in response to infection with *Verticillium dahliae*. In this interaction, the mRNA levels for the TCTP were lower in inoculated plants (96 h.a.i.) in relation to mock control plants. In rice, Zhao et al. (2008) [34] detected increased levels of mRNA for this same protein 12 h.a.i. with *Rhizoctonia solani*. By a proteomic approach, Liao et al. (2009) [35] detected increased levels of TCTP in leaves of two isogenic lines of rice (C101A51 and CO39, incompatible and compatible types, respectively) twelve hours after the application of the elicitor CS B I, purified from ZC13, a race of the rice blast fungus *Magnaporthe grisea*. The levels of this protein are highly regulated in response to a wide variety of extra-cellular signals and cellular conditions [36]. Several studies found changes in TCTP transcript or protein levels under diverse physiological conditions, such as light, aluminum stress, cold stress, *Agrobacterium tumefaciens*-mediated transformation, egg cell fertilization, or water deficit [37-42]. TCTP operates in various cellular processes like microtubule organization and ion homeostasis [36]. Studies have mentioned that it is capable of binding with tubulin as well as with calcium [43-45]. It is synthesized in mitotically active tissues, showing reduced accumulation in cells that are not actively dividing [46]. According to Li et al. (2001) [47], this protein has also anti-apoptotic functions. Programmed cell death (PCD) is an orderly process of cellular suicide that requires active gene expression [48]. In plants, it has been suggested that PCD is part of the plant response to pathogen invasion, causing the formation of a localized lesion of dead cells that limits cell-to-cell transfer of the pathogen in a process known as the HR (hypersensitive reaction) [49,50]. The reduced level of TCTP found in leaf cells of the genotype PI561356 72 h.a.i. with *P. pachyrhizi* found in this study, evidences the occurrence of HR by the PCD bearing in mind the functions proliferative and anti-apoptotic of the TCTP. We could speculate that a lower level of TCTP as a response to inoculation could be a physiological and cellular response to induce apoptosis in sites near injuries caused by the pathogen.

In the present study, the enzyme fructose-bisphosphate aldolase, which is part of the pentose phosphate pathway, showed increased levels in plants of genotype PI561356 72 h.a.i.. In fact, the spot corresponding to this enzyme was not detected

Table 1. Differentially accumulated proteins in soybean leaves in response to *Phakopsora pachyrhizi* inoculation. Genotypes PI561356 (spots 1 to 9) and EMBRAPA 48 (spots 10 to 12).

spot ID	Protein names	PMF protein score/database	identified peptides (PFF)	access number/ database	sequence coverage (%)	experimental Mr (kDa)	theoretical Mr (kDa)	pI
1	Translationally controlled tumor protein	85/NCBI (<i>Glycine max</i>)	MLVYQDLTGDLELSDSFR VVDIVDTFRLRQEPAFDKK LDAEQQLFKK DGAADPTFLFYALK GAVDIDIGANPSAEGGEGEDVDDAAVK FVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	gij20140688/ NCBI	55	21.560	19.098	4.57
2	Gamma glutamyl hydrolase	90/SwissProt (<i>Glycine max</i>)	YVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	P93164.1/ SwissProt	35	35.597	37.824	6.08
3	small subunit ribosomal protein S1	109/Phytozome (<i>Glycine max</i>)	YVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	Glyma17g04190.1	31	44.510	45.222	5.14
4	Fructose biphosphate aldolase	105/NCBI (<i>Glycine max</i>)	YVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	gi366500825 NCBI	27	39.423	32.945	6.73
5	Elongation factor 1-alpha	76/NCBI (<i>Ricinus communis</i>)	YVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	gi223532558/ NCBI	29	54.275	49.746	9.15
6	Glutamine synthetase	90/NCBI (<i>Glycine max</i>)	YVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	gi13877511/ NCBI	30	47.703	47.946	7.63
7	Rubisco subunit beta ligation protein	113/NCBI (<i>Ricinus communis</i>)	YVSGGARVPIIYN ESPENLNK NDAGDHPF VAFNLGGNIVIR FPSDLLTOLKTDCLVHNRHRYAISPR LSDFFEILATSGDR TFVSTAR NAFEWATSLKAPHTEDAIR KLFEAYER GTVFYTDINNGAVVDITAK SSAYLPLQEAQIHR SLQFDLAWER GGVAELEGRL FVEDEEGSRLVLSNR VTDISTVLQPGDILKVMILSHDR AEEMAGTFR IAQAEAMAR KPWSLSFSFGR	gi1157337766 NCBI	31	62.228	64.583	5.80

Mr (Molecular mass); pI (isoelectric point).

continued Table 1. Differentially accumulated proteins in soybean leaves in response to *Phakopsora pachyrhizi* inoculation. Genotypes PI561356 (spots 1 to 9) and EMBRAPA 48 (spots 10 to 12).

spot ID	Protein names	PMF protein score/database	identified peptides (PFF)	access number/ database	sequence coverage (%)	experimental Mr (kDa)	theoretical Mr (kDa)	pI
8	Rubisco subunit beta ligation protein	94/NCBI (<i>Pisum sativum</i>)	DLINILEDAIR NLIEAAEQEYEK GYISPYFVTDSEK NLIEAAEQEYEK AAVEEGIWGGGCTLLR SADNSLYVVEGMQFDR SQYLDIAILTGGTVIR DTTIVGDGSGTQEAANKR LGGVAVIQVGAQTETELK KSQYLDIAILTGGTVIR GYISPYFVTDSEK AAVEEGIWGGGCTLLR DLINILEDAIR NLIEAAEQEYEK GYISPYFVTDSEK NLIEAAEQEYEK AAVEEGIWGGGCTLLR SADNSLYVVEGMQFDR SQYLDIAILTGGTVIR DTTIVGDGSGTQEAANKR LGGVAVIQVGAQTETELK KSQYLDIAILTGGTVIR GYISPYFVTDSEK KITFAEYNAAFR	gi 2506277 NCBI	26	62.168	63.287	5.85
9	Rubisco subunit beta ligation protein	77/NCBI (<i>Pisum sativum</i>)	DLINILEDAIR NLIEAAEQEYEK GYISPYFVTDSEK NLIEAAEQEYEK AAVEEGIWGGGCTLLR SADNSLYVVEGMQFDR SQYLDIAILTGGTVIR DTTIVGDGSGTQEAANKR LGGVAVIQVGAQTETELK KSQYLDIAILTGGTVIR GYISPYFVTDSEK KITFAEYNAAFR	gi 2506277 NCBI	24	62.929	63.287	5.85
10	Glyceraldehyde 3-phosphate dehydrogenase subunit A	78/NCBI (<i>Glycine max</i>)	VIAWYDNEWGYSQR DLGIDLVEGTGVFVDR ESADNELQGLSVcDEPLVSVDFR IYCSVETWPR	gi 77540210/ NCBI	28	45.628	43.479	8.42
11	1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXP)	145/NCBI (<i>Pueraria montana</i> var. <i>lobata</i>)	LPLLYLSWPER ILPADSEHSAIFQSIQGLPK ELVASPSLEEIIHIDQWAR VCPSHVLDLFGPGEAFVWR VIAWYDNEWGYSQR VCPSHVLDLFGPGEAFVWR	gi 35187000/ NCBI	22	49.157	50.809	5.83
12	Carbonic anhydrase	114/NCBI (<i>Phaseolus vulgaris</i>)	VIAWYDNEWGYSQR VCPSHVLDLFGPGEAFVWR	gi 270342124/ NCBI	67	27.413	23.455	7.36

Mr (Molecular mass), pI (isoelectric point).

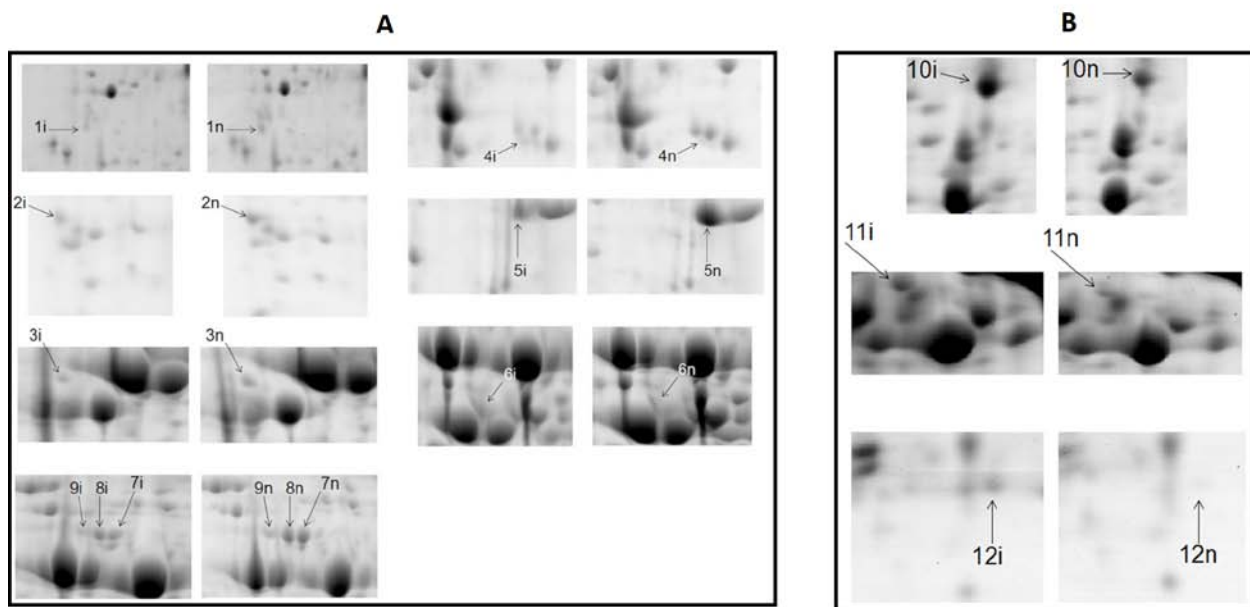


Figure 1. A: Spots corresponding to proteins differentially accumulated in partially resistant genotype PI561356 72 h.a.i. The symbols are as follows: 1i and 1n: tumoral protein translationally controlled; 2i and 2n: gamma glutamyl hydrolase; 3i and 3n: small subunit ribosomal protein S1; 4i and 4n: fructose bisphosphate aldolase; 5i and 5n: elongation factor 1-alpha; 6i and 6n: glutamine synthetase; 7i, 7n, 8i, 8n, 9i and 9n: Rubisco beta subunit binding protein. The indexes "i" and "n" indicate that the plants have been inoculated (i) or non-inoculated (n: mock) with *P. pachyrhizi*.

B: Spots corresponding to proteins differentially accumulated in susceptible genotype Embrapa 48 192 h.a.i. The symbols are as follows: 10i and 10n: glyceraldehyde-3-phosphate dehydrogenase subunit A; 11i and 11n: 1-deoxy-D-xylulose-5-phosphate-reductoisomerase (DXR); 12i and 12n: carbonic anhydrase. The indexes "i" and "n" indicate that the plants have been inoculated (i) or non-inoculated (n: mock) with *P. pachyrhizi*.

Table 2. Relative protein levels based on the spots mean %Volumes of inoculated (I) and non-inoculated plants (Mock) (N).

PI561356 (72 h.a.i.)				
spot ID	Protein putative identification	mean % volume±SD		accumulation level difference
		I	N	
1	Translationally controlled tumor protein	0.016±0.003	0.024±0.007	(-) 1.50x
2	Gamma glutamyl hydrolase	0.038±0.011	0.094±0.025	(-) 2.47x
3	small subunit ribosomal protein S1	0.030±0.006	0.064±0.017	(-) 2.13x
4	Fructose bisphosphate aldolase	0.019±0.009	ND	NA
5	Elongation factor 1-alpha	0.347±0.061	0.684±0.329	(-) 1.97x
6	Glutamine synthetase	0.226±0.097	0.160±0.049	(+) 1.41x
7	Rubisco beta subunit binding protein	0.066±0.015	0.235±0.062	(-) 3.56x
8	Rubisco beta subunit binding protein	0.082±0.023	0.236±0.067	(-) 2.88x
9	Rubisco beta subunit binding protein	0.041±0.015	0.093±0.015	(-) 2.27x
Embrapa 48 (192 h.a.i.)				
10	Glyceraldehyde-3-phosphate dehydrogenase subunit A	0.332±0.035	0.155±0.071	(+) 2.14x
11	1-deoxy-D-xylulose-5-phosphate-reductoisomerase (DXR)	0.061±0.012	0.024±0.014	(+) 2.54x
12	Carbonic anhydrase	0.060±0.002	ND	NA

The signals (+) and (-) indicate increase and decrease on protein synthesis as response to *P. pachyrhizi* inoculation, respectively. ND = not detected. NA = does not apply.

in mock plants. This might have been due to limitations of the 2D electrophoresis technique that tends not to allow detection of low abundance proteins [30]. It is also conceivable that the concentration of this enzyme in mock plants was below the limits

established for image analysis using the software ImageMaster. The pentose phosphate pathway is one of the main routes for the production of phenolic compounds [26], which are responsible for activation of defense mechanisms. Tremblay et al. (2010) [26]

showed that the levels of transcripts corresponding to proteins of this pathway were lower in soybean plants inoculated with *P. pachyrhizi* compared to mock plants. Fructose-bisphosphate aldolase was among these proteins. According to these authors their findings are in agreement with the fact that the susceptible cultivar they used (cv. Williams 82) could not build an efficient defense mechanism. Liao et al. 2009 [35] also found changes in the levels of fructose bisphosphate aldolase that showed down-regulated at 12 hours after the application of the elicitor CS B I (of the race ZC13 of the fungus *Magnaporthe grisea*) in leaves of both isogenic lines of rice C101A51 and CO39. Wang et al. (2012) [21] also detected increased levels of fructose-bisphosphate aldolase in a soybean resistant cultivar (SX6907) inoculated with *P. pachyrhizi* spores at 12 and 24 h.a.i..

Our data also showed decreased levels of a chloroplast ribosomal protein that is part of the 30S subunit (small subunit ribosomal protein S1) and of elongation factor 1-alpha in genotype PI561356 72 h.a.i.. Several studies on the biotrophic interactions between fungus and host plant show that the translational activity and the ribosome biogenesis are reduced in the initial stages of the infection [51-55]. Yamamoto et al. (1976) [56] have shown that reduction in protein synthesis, in fact, can be detected between the initial infection stage up to three days after infection (72 h.a.i.).

The present study showed a reduced level of a binding protein to the Rubisco beta subunit in plants of genotype PI561356 72 h.a.i.. A reduction in the accumulation of the transcript corresponding to the Rubisco small subunit was also observed in cultivar Williams 82, 10 days after inoculation with *P. pachyrhizi* [26]. Panthee et al. (2007) [22] also verified a reduction in the same transcript 72 h.a.i. with *P. pachyrhizi* in genotype 5601T. According to Ellis & Van Der Vies (1988) [57], the chloroplast contains soluble proteins that can bind non-covalently to the small and large Rubisco subunits. These authors indicated that these binding proteins belong to a general class of proteins called "molecular chaperones" which are required for the correct assembly of certain oligomeric proteins such as Rubisco. Our findings might be related to the decreased photosynthetic rates that have been observed in infected plants [26]. Wang et al. (2012) [21] showed that the levels of photosynthesis-related proteins like the Rubisco large subunit were negatively affected upon inoculation with *P. pachyrhizi*. According to these authors the inactivation of anabolic and activation of catabolic pathways might be important to provide energy for plant defense against pathogens. In the present work binding proteins to Rubisco subunit beta, small subunit ribosomal protein S1 and Elongation factor 1-alpha were all reduced in soybean leaves of resistant genotype PI561356 inoculated with *P. pachyrhizi* at 72 h.a.i..

We observed increased levels of glutamine synthetase (GS) in plants of genotype PI561356 at 72 h.a.i.. The availability of nitrogen has a significant impact in the development of diseases in plants and GS is one of the key enzymes of the nitrogen metabolism in plant cells [58]. Low availability of nitrogen frequently increases the susceptibility of plants to diseases [59]. Besides, nitrogen limitation can induce pathogenicity related

genes in bacteria and pathogenic fungus [60]. In contrast to our observation, Tremblay et al. (2010) [26] verified a reduced accumulation of transcripts corresponding to this protein in the susceptible genotype Williams 82 10 days after inoculation. Pageau et al. (2006) [58] observed alterations in the transcription levels of the cytosolic GS (GS1) in tobacco leaves (*Nicotiana tabacum* L.) inoculated with viral and bacterial pathogens. Increased GS1 level was observed in foliar tissue of infected tobacco plants.

We detected decreased accumulation of gamma-glutamyl hydrolase in plants of genotype PI561356 at 72 h.a.i.. Using 2-D electrophoresis associated with MS, Krishnan et al. (2011) [61] identified gamma-glutamyl hydrolase as a part of the xylem sap in soybean plants. The xylem sap protein composition can be significantly altered by infection with pathogens and this can be detected at distant points of the infection site [62]. Pathogenicity related protein like peroxidases, chitinases and serino-proteases have been detected in great amounts in the xylem sap in several plant species [63]. A proteomic study was conducted by Subramanian et al. (2009) [64] to identify changes in protein accumulation in the soybean xylem sap in response to symbiotic (*Bradyrhizobium japonicum*) and pathogenic (*Phytophthora sojae*) interactions. As a result of the pathogenic interaction, the authors verified increased synthesis of several proteins in the xylem sap and a parallel inhibition on fungal development. Rep et al. (2002) [62] observed altered protein accumulation in tomato xylem sap when plants were infected with the fungus *Fusarium oxysporum*.

The enzyme carbonic anhydrase was highly accumulated at 192 h.a.i. in susceptible genotype Embrapa 48 plants inoculated with *P. pachyrhizi* (Figure 1B). Unexpectedly, this enzyme that performs important roles in the chloroplast metabolism was not detected in the mock plants. The explanations for this observation could be also related with limitations of the electrophoresis technique and the image analysis software as discussed previously for the fructose bisphosphate aldolase. Carbonic anhydrase is a metalloenzyme that catalyzes the CO₂/HCO₃⁻ inter-conversion. In plants the conversion of HCO₃⁻ to CO₂ supplies the Rubisco enzyme with substrate. In C₄ plants, HCO₃⁻ produced from CO₂ is fixed by PEPcarboxylase. In C₃ plants like soybean, carbonic anhydrase enables carbon diffusion between cytosol and stroma in the form of HCO₃⁻, converting it to CO₂ only at the moment of carbon fixation by the Calvin cycle [65].

There are several pieces of evidence showing that carbonic anhydrase is involved with molecular defense mechanisms of plants against pathogens. In plants, salicylic acid (SA) has an important role in the local and systemic defense responses [66]. It is synthesized as a response to biotrophic pathogens [67]. In tobacco leaves, Slaymaker et al. (2002) [66] reported the presence of one SA binding protein (SABP3). The chloroplast soluble fraction was purified by chromatography, and the SA binding activity increased 600 times in the fraction corresponding to SABP3. MS and sequencing data revealed that SABP3 corresponded to carbonic anhydrase.

Restrepo et al. (2005) [68] analyzed the variation in the gene expression of *Solanum tuberosum* in its compatible interaction with *Phytophthora infestans*. The authors observed a decrease in the accumulation of carbonic anhydrase throughout the post-inoculation period. The gene encoding this enzyme was silenced in *Nicotiana benthamiana* and in silenced plants there was a faster development of the pathogen, indicating that in the absence of this enzyme there is an increased susceptibility to *P. infestans*. Panthee et al. (2007) [22] found increased levels of transcripts for SA effector proteins in cultivar 5601T at stage V2 in response to *P. pachyrhizi*. These results, added to the ones found in the present study, indicate a possible function of carbonic anhydrase in the metabolic mechanisms involved in plant defense to pathogens.

In the present study, we noticed an increased DXR level in the susceptible genotype Embrapa 48 192 h.a.i.. In chloroplasts as part of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway DXR catalyzes the synthesis of MEP which is the precursor of isopentenyl diphosphate and its isomer dimethylallyl diphosphate. These compounds are essential for isoprenoid biosynthesis [69,70]. Isoprenoids perform important functions in many aspects of cellular metabolism, including photosynthesis (carotenoids and chlorophyll), respiration (ubiquinone), development regulation (gibberellic acid and abscisic acid) and defense against pathogens (phytoalexins) [71-73].

The genotype Embrapa 48 also presented increased levels of GAPDH α 192 h.a.i.. In the chloroplast, this enzyme is part of the carbon fixation pathway for glucose biosynthesis. Laxalt et al. (1996) [74], using Northern blot, observed increased accumulation of transcripts for cytosolic GAPDH subunit C (GAPDH c) in leaves and stalks of *Solanum tuberosum* L. inoculated with *P. infestans*. When eicosapentaenoic acid, an elicitor produced by *P. infestans*, was applied to the stalk the transcript levels for GAPDH c also increased and in parallel there was induction of the gene encoding for the enzyme hydroxymethylglutaryl coenzyme A reductase, which participates in the synthesis of isoprenoids (phytoalexins). In this same study the authors submitted leaf tissue to salicylic acid and again differential accumulation of transcripts for GAPDH c occurred.

During plant interaction with pathogens, the systemic acquired resistance (SAR) is induced as part of the defense reaction of the plant as a whole. This leads to increased levels of the reactive oxygen intermediates (e.g. H_2O_2) [75]. Zaffagnini et al. (2007) [76], examining the effects of redox modifications in the activity of the chloroplast isoform of GAPDH from *Arabidopsis thaliana* showed that this enzyme was inhibited by oxidants like H_2O_2 . It is plausible to speculate that the increased GAPDH α

levels observed in the present work in soybean leaves of plants inoculated with *P. pachyrhizi* is a compensatory response to the reduced activity of the enzyme due to accumulation of H_2O_2 as part of the acquired immune response. Wang et al. (2012) [21] also detected increased levels of this protein in soybean leaves inoculated with *P. pachyrhizi* at 12 and 24 h.a.i..

In conclusion, it is clear that most of the differentially accumulated proteins detected in the present study in the interaction between *Phakopsora pachyrhizi* and soybean genotypes participate in metabolic pathways involved in plant defense against pathogens. The differential accumulation of carbonic anhydrase and GAPDH α , for instance, indicates that SAR was activated upon pathogen inoculation. Carbonic anhydrase is a potential effector of salicylic acid, and GAPDH α activity is inhibited by reactive oxygen compounds that are accumulated during SAR. A decreased synthesis of TCTP could be indicative of a HR which has as one of its processes the PCD, since TCTP has functions proliferative and anti-apoptotic. Our study also detected some proteins with differential synthesis that are potentially involved with plant defense against pathogens, such as DXR, fructose-bisphosphate aldolase and glutamine synthetase that presented highest accumulation after inoculation. The reduced levels of small subunit ribosomal protein S1, elongation factor 1- α and binding protein to Rubisco subunit beta are in accordance with the idea that decrease in anabolism and increase in catabolism may generate useful energy that can be used during response to pathogen attack. The gamma-glutamyl hydrolase showed altered accumulation upon *P. pachyrhizi* inoculation. Although the specific functions of this protein in plant defense are still unknown, our findings and those already described in the literature indicate a possible role for this protein in metabolic pathways related to plant defense against pathogens. Our findings not only contribute to the understanding of soybean response to *P. pachyrhizi*, but also corroborate previous information on this important pathosystem.

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