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Graphical abstract

Unadapted and adapted to starvation *Acholeplasma laidlawii* cells induce different responses of *Oryza sativa*, as determined by proteome analysis

Vladislav M. Chernov *, Olga A. Chernova, Elena S. Medvedeva, Alexey A. Mouzykantov, Anastasia A. Ponomareva, Gulnara F. Shaymardanova, Oleg V. Gorshkov, Maxim V. Trushin

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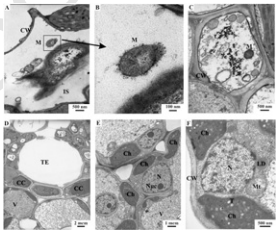

Features of ultrastructural organization of plants (*O. sativa* L.) cultivated in the medium with unadapted (A, B, C) and adapted (D, E, F) to UFE *A. laidlawii* PG8 cells. A — the leaf vascular system; B — intracellular space of the cancellous parenchyma; C — mycoplasma cell; D — the leaf vascular system; E — parenchyma of a leaf; F — a cells of cancellous parenchyma. Ch — chloroplast, CW — cell wall, IS — intercellular space, CC — cells of covering, LD — lipid drop, M — mycoplasma, Mt — mitochondria, N — nucleus, Nuc — nucleolus, T — thylakoids, TE — tracheal elementsand V — vacuole.

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Supplementary materials.

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Unadapted and adapted to starvation *Acholeplasma laidlawii* cells induce different responses of *Oryza sativa*, as determined by proteome analysis

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ABSTRACT

For the first time, we studied the phytopathogenicity toward *Oryza sativa* L. of unadapted and adapted to unfavorable environment (starvation) cells of *Acholeplasma laidlawii* PG8 — ubiquitous mycoplasma found in the soil, waste waters, tissues of the highest eukaryotes and being the basic contaminant of cell cultures and a causative agent of phytomycoplasmoses. The features of morphology, ultrastructural organization and proteomes of unadapted and adapted cells of the mycoplasma and infected plants were presented. Using 2D-DIGE and MS, 43 proteins of *O. sativa* L. that were differentially expressed in the leaves of plants cultivated in media with *A. laidlawii* PG8 were identified. The qualitative and quantitative responses of the plant proteome toward adapted and unadapted mycoplasma cells differed. That may be explained by differences in the virulence of the corresponding bacterial cells. Using 2D-DIGE and MS, 82 proteins that were differentially expressed in adapted and unadapted mycoplasma cells were detected. In adapted cells of the mycoplasma, in comparison with unadapted ones, a significant increase in the expression of PNPase — a global regulator of virulence in phytopathogenic bacteria occurred; there was also decreased expression of 40 proteins including 14 involved in bacterial virulence and the expression of 31 proteins including 5 involved in virulence was not detected. We propose that differences in the phytopathogenicity of adapted and unadapted *A. laidlawii* PG8 cells may be related to features of their proteomes and membrane vesicles.

1. Introduction

Acholeplasma laidlawii (class Mollicutes) is a unique mycoplasma from the viewpoint of its adaptation capabilities [1,2]. This mycoplasma is widely distributed in nature, one of the five common species of cell culture contaminants, and is a causative agent of some plant diseases [3–7]. Insects are considered to be mycoplasma vectors toward plants [8]. However, *A. laidlawii* can invade plants through the root system [9]. The ability of *A. laidlawii* to display virulence (infectivity, invasivity, toxigenicity and persistence) toward plants suggests that it can successfully survive unfavorable environment (UFE) such as oxidizing conditions, limited substrate and temperature fluctuations. We found [1,5,10]

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that the adaptation of *A. laidlawii* PG8 to starvation involves changes in morphology, ultrastructure, DNA topology, genotoxicity of the mycoplasma cells and virulence of the bacterium toward *Vinca minor* L. — the specific indicator of phytomycoplasmoses. Meanwhile, the molecular biology of the interaction of *A. laidlawii* with plants has not yet been studied. Proteomics is an efficient tool for investigating the plant–microbe interaction at the molecular level when the complete nucleotide sequences of the genomes of both host and pathogen are known. However, these kinds of reports regarding plants and mycoplasmas are very rare [11,12], and are totally absent for *A. laidlawii*.

In this study, for the first time, the morphology, ultrastructure and proteomes of *Oryza sativa* L. infected with cells of *A. laidlawii* PG8 that were unadapted and adapted to UFE (starvation) were investigated. Phytopathogenicity of *A. laidlawii* PG8 toward *O. sativa* L. was demonstrated, and it was found that the virulence of mycoplasma cells that were unadapted and adapted to UFE differed. Unadapted and adapted to starvation *A. laidlawii* cells induced different responses of *O. sativa*, as determined by proteome analysis.

2. Materials and methods

2.1. Bacterial strain, culture conditions

A. laidlawii PG8 strain obtained from the N.F. Gamaleya Institute of Epidemiology and Microbiology (Moscow) was used in this work. The mycoplasma cells were grown for 22–24 h in a liquid modified Edward medium. The complete medium contained the following components (%): tryptose, 2; NaCl, 0.5; KCl, 0.13; Tris, 0.3; horse blood serum, 10; yeast extract, 5; glucose, 1; penicillin, 1000 U/ml; and phenol red, 0.3 ml of 1% solution [1]. Glucose, yeast extract, and serum were excluded in case of UFE (starvation). To adapt *A. laidlawii* PG8 to the unfavorable growth conditions, the cells were harvested at the logarithmic growth stage (22–24 h at 37 °C), stored for 2 h at 8 °C, collected by centrifugation (4500 ×*g*, 8 °C, 20 min), resuspended in four volumes of the limited medium, and then stored at 30 °C for 8 weeks. *A. laidlawii* PG8 cells grown in the limited medium – adapted to UFE –, while grown in the Edward's medium – unadapted to UFE – were of control one [1]. Investigations were performed for *A. laidlawii* PG8 cells of 22–24 h (exponential phase growth) in case of unadapted culture and of 8 weeks in case of adapted to UFE mycoplasma.

2.2. Inoculation of plants with *A. laidlawii* PG8

Rice seeds (*O. sativa* L. breed "Lougovoy") were sterilized with 0.01% solution of $KMnO_4$ for 30 min, and then washed extensively with distilled water. The plants were grown in sterile conditions [13] at 27 °C (12 h:12 h light:dark photoperiod and a light intensity 6 klux). Rice plants were infected with *A. laidlawii* PG8 cells under sterile conditions as described by Chernov et al. [5] using a spontaneous infection of 10-day plant seedlings through the root system. Plant roots were incubated continuously in buffer (6.94 mM $Ca(NO_3)_2$; 3.09 mM KNO_3; 2.2 mM KCl; 2.15 mM KH_2PO_4; 2.08 mM $MgSO_4$, 1.4 mM KH_2PO_4; 10 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl; pH 7.2) containing *A. laidlawii* PG8 cells. Control plants were incubated with the mycoplasma-free buffer. Analysis of the samples was performed 7 days later.

2.3. Transmission electron microscopy and atomic force microscopy

Transmission electron microscopy was done according to Cole [14]. Material was fixed with 2.5% glutaraldehyde ("Fluka", Germany) in 0.1 M phosphate buffer (pH 7.2) for 2 h. Then, the material was dehydrated using an acetone, ethanol and propylene series and post-fixed in 0.1% OsO_4 with the addition of 25 mg/ml of saccharose. After treatment with epoxy resin ("Serva", Switzerland), ultra thin sections were obtained using LKB-III ultramicrotome (Sweden) and then were stained with uranyl acetate (for 10 min) and lead citrate (for 10 min) and examined using a JEM-1200EX transmission electron microscope ("Joel", Japan).

The probe microscopy of *A. laidlawii* PG8 cells was done according to Braga and Ricci [15]. Cells of *A. laidlawii* PG8 were centrifuged at 18,000 ×*g* for 30 min. The pellet was washed with phosphate-salt buffer (PBS, pH=7.2). A cell suspension was placed onto mica surface, 1 min later the sample was washed twice with redistilled water, and then air-dried (~10 min). Cells were examined using a Solver P47H atomic force microscope ("NT-MDT", Russia). fpN11S cantilevers (r=10 nm, Advanced Technologies Center, Russia) were used to scan the samples in the tapping mode. Nova 1.0.26 RC1 software ("NT-MDT", Russia) was used for image processing.

2.4. Proteins preparation for 2D-DIGE analysis

Proteins isolated from leaves of the plants grown in media with and without *A. laidlawii* PG8 cells were designated experimental and control samples, respectively. Proteins were independently extracted three times from different batches of leaf samples as described in Wang et al. [16]. Leaf tissues (1.5 g) were ground to a fine powder with a pestle in liquid nitrogen and then pre-chilled acetone with 10% v/v TCA was added. Samples were stored overnight at –20 °C and then centrifuged at 12,000 ×*g* for 15 min at 4 °C. The supernatant was precipitated with pre-chilled acetone with 10% v/v TCA for 2 h and then centrifuged 12,000 ×*g* for 15 min. The pellets were washed with pre-chilled acetone, incubated at –20 °C for 2 h and centrifuged again at 12,000 ×*g* for 15 min at 4 °C. The washing was repeated three times. The final pellets were lyophilized.

Proteins from cells of *A. laidlawii* PG8 were isolated as described in Demina et al. [17]. Cells were collected by centrifugation at 18,000 ×*g* for 30 min. The pellet was washed twice, first with a buffer containing 150 mM NaCl, 50 mM Tris–HCl, and 2 mM $MgCl_2$ at pH 7.4, and then in the same buffer containing in addition 1.38 mM PMSF. After cell treatment with Nuclease Mix (GE Healthcare, UK), proteins were dissolved in the following buffer: 8 M urea, 2 M thiourea and 16.7% solution (30% CHAPS+10% NP-40). The samples then were centrifuged at 15,000 ×*g* for 15 min.

Protein concentrations in the samples were measured by the Bradford method using "Quick Start Bradford dye" ("Bio-Rad").

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2.5. Cy-Dye labeling, IEF and 2D-DIGE

Protein staining with fluorescent dyes — CyDye-DIGE Cy3 (GE Healthcare, UK) for the control culture and CyDye-DIGE Cy5 (GE Healthcare, UK) for the experimental culture was performed in agreement with recommendations of the manufacturer (400 pmol per 50 μg of total protein) via incubation in the dark for 30 min at +4 °C. Staining reaction was stopped with 10 mM solution of lysine. One-dimensional electrophoresis in 12% polyacrylamide gel was then done to check the efficacy of staining. Before isoelectrofocusing, the samples were mixed in equimolar proportions, and DTT added to give a final concentration of 80 mM and ampholines 3–10 to give a final concentration of 0.2% were added. Experiments were repeated at least three times.

Isoelectrofocusing was performed in 18 cm glass tubes in 4% polyacrylamide gel (8 M urea, 4% acrylamide/methylene bis-acrylamide, 2% ampholines (pH 3–10), 4% ampholines (pH 5–8), 6% solution containing 30% CHAPS and 10% NP-40, 0.1% TEMED, 0.02% ammonium persulfate). Isoelectrofocusing was done in the following regime: 100 V–200 V–300 V–400 V–500 V–600 V — for 45 min, 700 V — for 10 h, 900 V — for 1 h. After finishing isoelectrofocusing, tubes were equilibrated for 30 min in a buffer containing 6 M urea, 30% glycerol, 62.5 mM Tris–HCl (pH 6.8), 2% SDS, bromphenol blue and 20 mM DDT. Then tubes were placed onto surface of gradient (9–16%) polyacrylamide gel and fixed with 0.9% agarose with bromophenol blue. Electrophoresis was performed in Tris–glycine buffer with cooling at the following regime: 20 mA per glass — for 20 min, 40 mA per glass — for 2 h, 35 mA per glass — for 2.5 h.

2.6. Gel analysis

The electrophoregrams were scanned using Typhoon Trio scanner (GE Healthcare, UK) at laser wavelengths of 532 nm (green fluorescence) and 633 nm (red fluorescence). The data were analyzed with Phoretix 2D Advanced v6.01 software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Carrying out the IEF we used IEF standards (Bio-Rad). Analyzing 2D gels, knowing the localization areas of protein standards we calibrated (in Phoretix 2D Advance software) the tubes according to them. Spots that coincided, at least, in duplicate were selected for subsequent comparison and identification. Data were presented as mean and standard deviation. A cut-off value was set at a 1.5-fold increase or decrease, and differences in spot intensities were analyzed by Student's t-test with $p<0.05$.

2.7. In-gel digestion

Proteins were hydrolyzed and extracted from the gel using the protocol described in [18]. Protein spots were excised from gel and washed in 150 μl 50 mM NH_4HCO_3 in 30% (v/v) acetonitrile. To dry pellets, 7 μl of trypsin solution (6.25 ng μl^{-1}, "Promega", USA) in 20 mM NH_4HCO_3 was added. They were then incubated at 4 °C for 45 min, and then overnight at 37 °C. After hydrolysis, gel fragments were dried at centrifugal desiccator, washed in 20 μl of deionized water and then dried again. To extract peptides, a 20 μl 0.1% (v/v) solution of trifluoroacetic acid in deionized water was added to gel fragments that were then incubated for 20 min at room temperature. Supernatant was sampled into separate tubes. For repeated extraction, 20 μl of a 0.1% (v/v) solution of trifluoroacetic acid in 50% acetonitrile was added, and then samples were incubated for 40 min at room temperature. This supernatant was combined with the first one, and then concentrated in a centrifugal desiccator to 1–2 μl; after that, 5 μl of 0.1% solution of trifluoroacetic acid in 50% acetonitrile was added, and the resulting. Samples analyzed with mass-spectrometry.

2.8. Protein identification

Mass-spectra were obtained using a MALDI-TOF/TOF Ultraflex II mass-spectrometer ("Bruker", USA), with a UV-laser in regime of the positive ions in diapason 500–4000 Da using reflectron. The proteins were identified from the masses of proteolytic fragments using Mascot Peptide Fingerprint (Matrix Science, USA) software and NCBI databases [http://www.ncbi.nlm.nih.gov]. For *O. sativa* proteins, search was made in NCBI database among proteins of all organisms while for *A. laidlawii* — among proteins of *A. laidlawii*. Search parameters for MASCOT: enzyme trypsin; a number of erroneous restriction sites — 1; database — NCBInr; Taxonomy — *O. sativa* L. for rice, to search proteins of *A. laidlawii* PG8, local server was used that contained amino acid sequences of all mycoplasma proteins in FASTA format downloaded from NCBI FTP-server (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Acholeplasma_laidlawii_PG_8A_uid58901); variable modifications for rice — acetyl (N-term), Oxidation (M), Propionamide (C), variable modifications for *A. laidlawii* PG8 — Oxidation (M); Propionamide (C). MH^+ ion mass identification accuracy was 0.005%; possible modifications of cysteine residues by acrylamide and methionine oxidation were taken into consideration. In some cases use has been made of MS/MS data for peptide

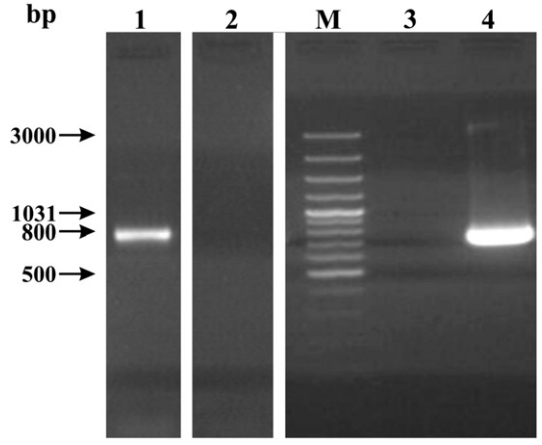

Fig. 1 – Electrophoregrams of amplification products of the nucleotide sequence of *trx*-gene of *A. laidlawii* PG8 in plants infected with unadapted (1) and adapted (2) to UFE cells of the mycoplasma, 3 and 4 — results of amplification of the reaction mix without mycoplasma DNA and with DNA of *A. laidlawii* PG8 (negative and positive control, respectively), M — marker of a length of fragments.

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fragmentation analysis followed by Blast searches (to find the corresponding note, it is necessary to enter a number of sequences without characters and symbols to search line of NCBI start page (http://ncbi.nim.nih.gov)). Protein scores >44 and 83 were considered significantly matched for *A. laidlawii* PG8 and *O. sativa* L., respectively. Three independent biological replicates were made in proteomic analysis for all cases. Since up- and down-regulated isoforms of the same proteins have been registered in some cases, to avoid misunderstanding, all isoforms were included in the total number of differentially expressed proteins.

2.9. *Polymerase chain reaction and product analysis*

Directed amplification of *A. laidlawii* PG8 DNA fragments via the polymerase chain reaction (PCR) was done using primers synthesized on the basis of the known nucleotide sequences for *trx* gene (F: 5′-ggcaagaaggcgaaggttt-3′; R: 5′-gcctgtggtgtcatctgtatc-3′). Oligonucleotides were synthesized in Litech Co. Ltd (Moscow, Russia). PCR was performed in the reaction mix consisting of 1×PCR buffer (100 mM Tris–HCl (pH 8.7), 50 mM KCl, 2 mM $MgCl_2$), mix of deoxyribonucleoside triphosphate, 400–500 ng of DNA matrix and 0.02 ng of each primer. Taq-polymerase ("Litech", Russia) was added to the reaction mix before beginning the reaction in concentrations recommended by the producer. The final volume of the reaction mix was 25 μl. Mineral oil was thickened on the reaction mix. The following cycling conditions were used: 95 °C for 3 min and 40 cycles at 95 °C for 30 s, 63 °C for 30 s, 72 °C for 10 s and 72 °C for 10 min. The reaction regimen was controlled with the use of "Tercyc" amplifier ("DNA-Technology", Russia).

The analysis of the PCR products was performed using electrophoretic separation of the DNA fragments in 2% agarose gel ("Helicon", Russia) and consequent staining with ethidium bromide (10 mg ml^{-1}) and analyzed using gel documentation ("Dia-M", Russia). Both normal and abnormal

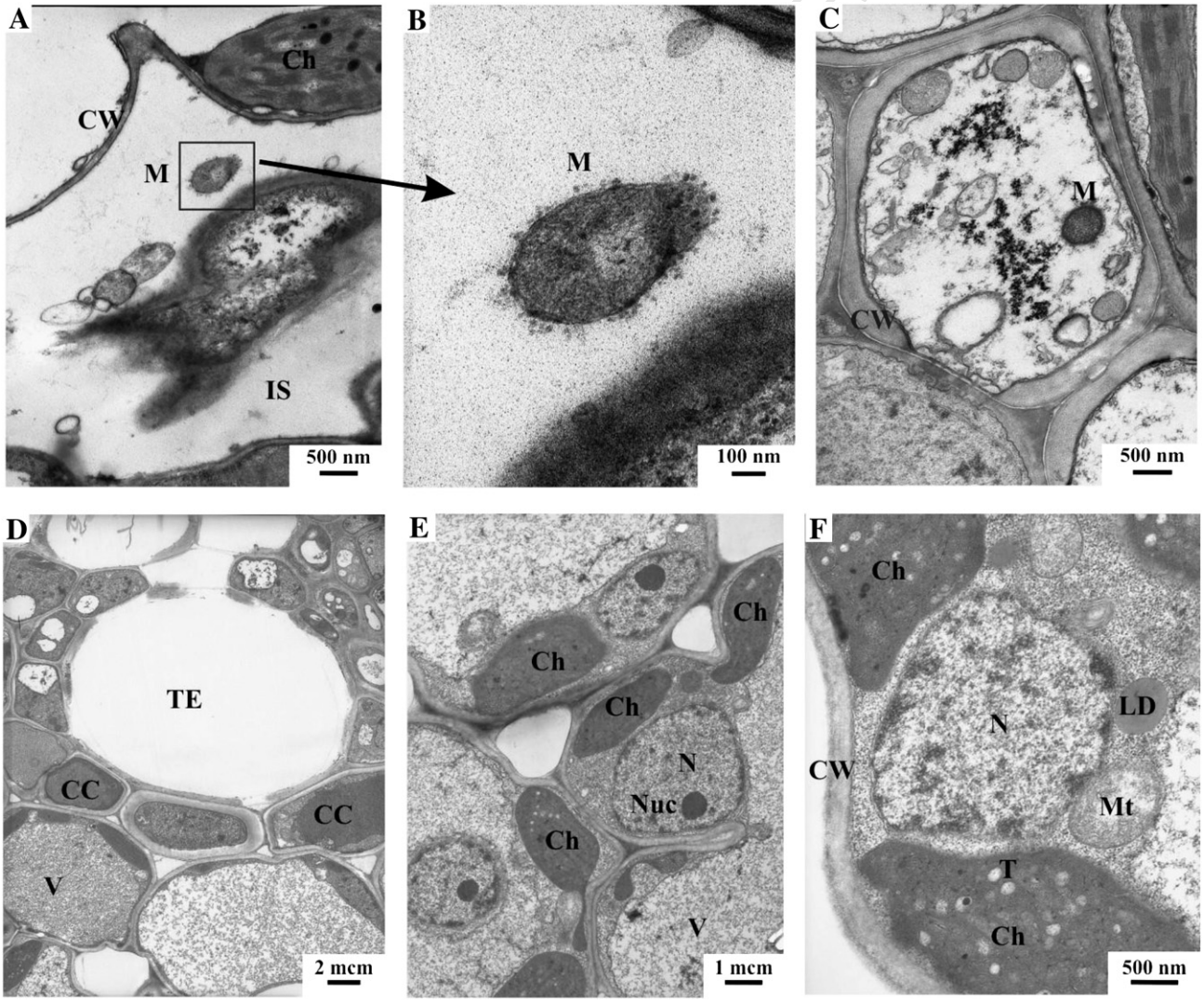

Fig. 2 – Features of ultrastructural organization of plants (*O. sativa* L.) cultivated in the media with unadapted (A, B, C) and adapted (D, E, F) to UFE *A. laidlawii* PG8 cells. A — the leaf vascular system; B — intracellular space of the cancellous parenchyma; C — mycoplasma cell; D — the leaf vascular system; E — parenchyma of a leaf; F — a cells of cancellous parenchyma. Ch — chloroplast, CW — cell wall, IS — intercellular space, CC — cells of covering, LD — lipid drop, M — mycoplasma, Mt — mitochondria, N — nucleus, Nuc — nucleolus, T — thylakoids, TE — tracheal elements, V — vacuole.

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leaves of *O. sativa* L. were used for PCR analysis. Leaves without visual morphological abnormalities were used for DIGE.

2.10. *RT-PCR analysis*

RNA isolation from cells of *O. sativa* L. tissues was performed with the use of commercial kit SV Total RNA Isolation System (Promega, USA). RNA isolation was done in triplicate.

NanoDrop ND 1000 ("Thermo Fisher Scientific Inc.", USA) and electrophoresis in 0.8% agarose gel with ethidium bromide (10 mg ml^{-1}) were used to check the purity of the extracted RNA. In reactions, RNA with $A_{260}/A_{280}=2.0$ value was used. RNA samples were stored at −20 °C.

Reaction of reverse transcription was performed with oligo $(dT)_{18}$ primers according to recommendations by producer of RevertAid™ M-MuLV reverse transcriptase ("Fermentas", Lithuania) after preliminary leveling of RNA concentration between samples. Amplification of RNA samples with specific primers was used to control DNA residual in RNA preparation.

To amplify genes for UPP (BAG87598), TufA (AAL37431), RASIPs (AAX95414), MAT, putative methionine adenosyltransferase (BAC65881) of *O. sativa* L., we used oligonucleotide primers produced by SPC Litekh (Moscow, Russia) on the basis of corresponding nucleotide sequences: (UPP: F1, 5′-gccaggcacccaagtaca-3′, R1, 5′-ccttgattccaccgcagc-3′; EF-Tu: F2, 5′-gcggcatcaccatcaacacc-3′, R2, 5′-tgtgctctttggtctgcggc-3′; RASIPs: F3, 5′-aggtgacatcggcggtgaac-3′, R3, 5′-cctggtgatgtcctgctggt-3′; MAT: F4, 5′-accgaggtccgcaagaac-3′, R4, 5′-gggttgaggtggaagatggt-3′).

PCR was performed in 25 μl volume of 600 μl centrifuge tubes on the programmed amplifier "Tercyk" ("DNA-technology", Russia) in the following regime: 1 cycle–95 °C–3 min.; 25 cycles–94 °C–10 s, 55 °C–5 s, 72 °C–5 s; 1 cycle–72 °C–2.5 min.

Reaction mix (25 μl) included the following components: 100 mM Tris–HCl (pH 8.3), 50 mM KCl, 2–3 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of *Taq*–polymerase ("Litekh", Russia), 20 pmol of each primer. Mineral oil was thickened on the reaction mix.

The analysis of the PCR products was performed using electrophoretic separation of the DNA fragments in 2% agarose gel ("Helicon", Russia) and consequent staining with ethidium bromide (10 mg/ml) and analyzed using gel documentation ("Dia-M", Russia).

The intensity of line after electrophoretic separation of amplification products was quantitatively assessed using Phoretix 1D software (v. 2003.02) (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Gene expression was expressed as relation of signal intensity from amplicon obtained on the basis of cDNA sequence of gene in experiment (Oi) to intensity from amplicon in control (Ki).

2.11. *Bioinformatic analyses*

BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and Pfam (http://www.sanger.ac.uk/Software/Pfam/) software were used for analyses of domains in amino acid sequences of proteins.

3. Results and discussion

Analysis of the morphology of plants grown in media with unadapted to UFE *A. laidlawii* PG8 cells showed that cells of the mycoplasma culture did not cause morphological abnormalities characteristic of phytomycoplasmoses (dwarfism, development of the lateral bines, growth inhibition, chlorosis). Only in some plants apical necrosis, chlorosis and leaf twisting were present. However, results of PCR analyses confirmed the presence of *A. laidlawii* PG8 DNA in the tissues of leaves from plants grown in the medium with unadapted to UFE mycoplasma cells (Fig. 1).

Transmission electron micrographs prepared from infected plants showed the presence of single membrane-

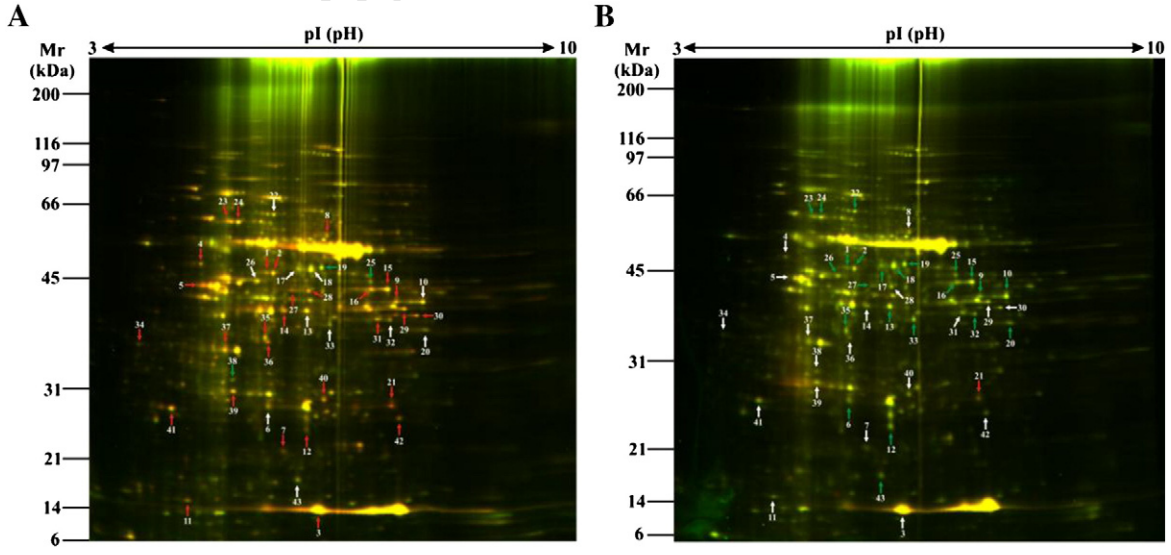

Fig. 3 – 2D-electrophoregrams of the proteins from leaves of *O. sativa* L. plants grown in media with unadapted to UFE *A. laidlawii* PG8 (A) and adapted ones (B). Arrows and numerals indicate the differently expressed plant proteins (Table 1). Proteins of non-infected plants are stained with green, infected — with red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 1 – The differentially expressed proteins from leaves of plants grown in media containing adapted to UFE *A. laidlawii* PG8 cells and unadapted ones.

Spot no[1].	Protein name	Score	SC (%)[2]	Number of mass values searched	Number of mass values matched	Number of mass values no matched	NCBI accession number[3]	Theoretical Mr (Da)/pI[4]	Experimental Mr (Da)/pI[5]	Fold change[6]	
										UAC[7]	AC[8]
1	2	3	4	5	6	7	8	9	10	11	12
1	Elongation factor Tu (TufA)	241	48	29	21	8	gi|17225494	50,666/6.19	46,733/5.53	1.606± 0.119	−1.79± 0.390
2	Elongation factor Tu (TufA)	208	36	19	16	3	gi|17225494	50,666/6.19	46,541/5.54	2.27± 0.509	−1.608± 0.077
3	Ribulose bisphosphate carboxylase small chain c (RuBisCO_small)	128	70	20	9	11	gi|149392567	12,238/8.46	12,782/6.08	1.686± 0.226	NC
4	Ribulose bisphosphate carboxylase/oxygenase activase (RA)	160	36	17	13	4	gi|109940135	51,848/5.43	49,045/5.41	1.543± 0.045	NC
5	RuBisCO activase small isoform precursor (RASIPs)	119	25	14	10	4	gi|62733297	52,478/5.59	43,975/5.43	2.931± 0.764	NC
6	23 kDa polypeptide of chloroplast photosystem II (PsbP)	94	39	7	5	2	gi|164375543	20,088/5.56	26,111/5.53*	NC	−1.503± 0.159
7	Putative Photosystem I reaction center subunit IV (PsaE)	176	46	4	2	2	gi|34394725	15,537/9.64	21,601/5.57	2.096± 0.169	NC
8	ATP synthase CF1 alpha subunit (AtpA)	375	53	33	28	5	gi|11466784	55,701/5.95	56,944/6.13	1.776± 0.514	NC
9	Glyceraldehyde-3-phosphate dehydrogenase, chain O (GAPDH)	88	23	23	9	14	gi|256032543	36,472/6.67	41,130/6.5	1.538± 0.060	−1.667± 0.071
10	Glycer aldehyde-3-phosphate dehydrogenase, chain O (GAPDH)	138	31	18	11	7	gi|256032543	36,472/6.67	40,603/6.61*	NC	−1.663± 0.156
11	Thioredoxin M-type (TrxM)	101	20	3	2	1	gi|11135471	18,517/8.16	14,700/5.19	1.811± 0.248	NC
12	Germin-like protein I (Glp)	167	20	4	2	2	gi|4239821	21,846/6.01	23,370/5.99*	1.5± 0.459	−1.526± 0.203
13	Malate dehydrogenase (MDH)	132	57	29	11	18	gi|110289264	23,670/6.21	38,983/5.98*	NC	−1.556± 0.074
14	Malate dehydrogenase (MDH)	102	55	41	10	31	gi|110289264	23,670/6.21	39,878/5.57	2.13± 0.533	NC
15	Nad-dependent formate dehydrogenase (Fdh)	220	43	30	18	12	gi|4760553	41,501/6.87	43,292/6.45	1.551± 0.070	−1.502± 0.118
16	Nad-dependent formate dehydrogenase (Fdh)	130	24	17	10	7	gi|4760553	41,501/6.87	43,292/6.38	1.945± 0.584	−1.521± 0.094
17	Methionine adenosyltransferase (MAT)	108	26	14	8	6	gi|3024122	43,442/5.68	46,336/5.81*	NC	−2.495± 0.077
18	Methionine adenosyltransferase (MAT)	158	38	19	12	7	gi|3024122	43,442/5.68	46,336/6.02*	NC	−2.016± 0.045
19	Methionine adenosyltransferase (MAT)	251	53	20	16	4	gi|17529621	43,760/5.93	46,718/6.08*	−2.26± 0.520	−2.138± 0.193
20	Putative lipase (Lip)	109	27	11	8	3	gi|55296706	37,110/8.22	36,206/6.62*	NC	−1.571± 0.111
21	Unnamed protein product (UPP)	219	62	21	16	5	gi|215686337	22,573/6.21	26,333/6.47*	1.723± 0.108	1.792± 0.250

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t1.27 **Table 1** *(continued)*

t1.28 Spot no[1].	Protein name	Score	SC (%)[2]	Number of mass values searched	Number of mass values matched	Number of mass values no matched	NCBI accession number[3]	Theoretical Mr (Da)/pI[4]	Experimental Mr (Da)/pI[5]	Fold change[6]	t1.29
										UAC[7]	AC[8]
t1.30 1	2	3	4	5	6	7	8	9	10	11	12
t1.31 22	Hypothetical protein (OsI_04213)	121	20	19	11	8	gi| 218189276	61,008/5.36	61,227/5.54	NC	−1.815± 0.165
t1.32 23	Hypothetical protein (Os06g0114000)	215	27	18	16	2	gi| 115466004	64,401/5.60	59,700/5.47*	2.690± 0.553	−1.656± 0.210
t1.33 24	Hypothetical protein (Os06g0114000)	302	39	25	22	3	gi| 115466004	64,401/5.60	59,509/5.48*	1.691± 0.081	−1.532± 0.118
t1.34 25	Hypothetical protein (Os01g0654500)	149	28	19	12	7	gi| 115438939	46,440/6.34	44,190/6.36*	−1.594± 0.219	−1.510± 0.163
t1.35 26	Hypothetical protein (Os11g0163100)	161	44	21	12	9	gi| 115484337	41,880/5.31	44,074/5.51*	NC	−1.958± 0.516
t1.36 27	Hypothetical protein (Os03g0129300)	125	27	18	10	8	gi| 115450493	47,650/6.22	42,454/5.66*	2.267± 0.094	−1.524± 0.221
t1.37 28	Hypothetical protein (Os03g0129300)	140	30	30	13	17	gi| 115450493	47,650/6.22	43,065/6.01	1.648± 0.480	NC
t1.38 29	Hypothetical protein (Os04g0459500)	128	28	19	10	9	gi| 115458768	43,115/7.62	40,105/6.53	2.132± 0.111	NC
t1.39 30	Hypothetical protein (Os12g0420200)	297	51	23	19	4	gi| 115488340	41,649/8.59	38,967/6.59	2.115± 0.350	NC
t1.40 31	Hypothetical protein (Os03g0327600)	118	29	17	9	8	gi| 115452789	39,366/6.30	38,398/6.41	1.55± 0.207	NC
t1.41 32	Hypothetical protein (Os03g0327600)	267	54	22	18	4	gi| 115452789	39,366/6.30	37,710/6.46*	NC	−1.506± 0.017
t1.42 33	Hypothetical protein (Os01g0649100)	123	20	11	8	3	gi| 115438875	35,723/8.74	36,785/6.13*	NC	−1.51± 0.072
t1.43 34	Hypothetical protein (Os07g0614500)	87	27	21	7	14	gi| 115473331	24,918/4.36	34,186/4.34	1.520± 0.009	NC
t1.44 35	Hypothetical protein (Os08g0191700)	106	29	11	8	3	gi| 115475151	32,959/5.51	35,325/5.53	1.506± 0.126	−1.769± 0.350
t1.45 36	Hypothetical protein (Os02g0133800)	157	48	15	10	5	gi| 115444057	29,967/5.37	34,528/5.53	3.02± 0.801	NC
t1.46 37	Hypothetical protein (Os01g0501800)	237	55	25	16	9	gi| 115436780	35,124/6.10	33,276/5.46	1.887± 0.304	NC
t1.47 38	Hypothetical protein (Os07g0694700)	161	49	17	12	5	gi| 115474285	27,243/5.21	29,345/5.47	−2.252± 0.008	NC
t1.48 39	Hypothetical protein (OsJ_30136)	168	50	17	11	6	gi| 125606445	26,349/8.78	28,067/5.48	1.836± 0.227	NC
t1.49 40	Hypothetical protein (Os03g0169100)	87	33	13	6	7	gi| 115450991	29,301/8.65	27,165/6.11	1.82± 0.015	NC
t1.50 41	Hypothetical protein (Os02g0537700)	117	27	10	7	3	gi| 115446541	28,364/5.67	26,187/4.90	1.787± 0.080	NC
t1.51 42	Hypothetical protein (Os07g0500300)	90	19	3	1	2	gi| 115472239	18,905/6.51	25,135/6.51	1.667± 0.176	NC
t1.52 43	Hypothetical protein (Os01g0675100)	145	55	12	8	4	gi| 115439131	17,351/5.58	17,266/5.81*	NC	−1.564± 0.130

Note: [1]A number of a spot on a gel; [2]sequence coverage; [3]identification number of a protein in NCBI database; [4]theoretical molecular weight and pI; [5]experimental molecular weight and pI detected in the study with unadapted and adapted (*) to UFE *A. laidlawii* PG8 cells; [6]mean ± standart deviation; [7]UAC — unadapted to UFE *A. laidlawii* PG8 cells; [8]AC — adapted to UFE *A. laidlawii* PG8 cells. NC — no changes in the expression between the control and experimental variant. t1.53

bound spherical cells with sizes of 500–700 nm characteristic of typical vegetative cells of the mycoplasma in the leaf conduction system and intracellular spaces of the cancellous parenchyma (Fig. 2A,B). Plants infected with the mycoplasma displayed changes of ultrastructure such as damaged integrity of the tonoplast, the presence of osmiophilic component in the central vacuole, reduction of the gran system as well as increasing a number of plastoglobules (Fig. 2A,C).

O. sativa L. grown in a medium with adapted to UFE mycoplasma cells also did not display the morphological abnormalities characteristic of phytomycoplasmoses in plants. As in the case of plants infected with unadapted to UFE mycoplasma, only some plants displayed chlorosis and leaf twisting but *A. laidlawii* PG8 DNA and the mycoplasma cells were not detected in the tissues of *O. sativa* L. when PCR and electron microscopy were used. Nevertheless, significant

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changes were detected in the ultrastructure of leaves of the plants (Fig. 2D–F): chloroplasts were located along the cell walls and did not contain amyloid grains; in the background of the electron dense stroma of chloroplasts there was a more transparent area of intra-thylakoid space; the gran system was poorly developed; mitochondrial matrix was with rare cristas.

Using a proteomic approach we identified proteins of *O. sativa* L., differentially expressed in plants infected with adapted and unadapted *A. laidlawii* PG 8 cells. About 700 protein spots were resolved in the analytic gel. Probably, the low resolution is connected with more broad pH range of IPG we used (3–10) then it is mainly used in the plant proteomic study (3–7).

In a 2D-DIGE of the proteins from leaves of *O. sativa* L. we identified 43 proteins differentially expressed in the plants cultivated in media with the mycoplasmas, 32 and 24 from unadapted and adapted to UFE mycoplasmas, respectively (Fig. 3; Suppl_Fig. 1, Table 1). The differentially expressed proteins of *O. sativa* L. participate in the responses of plants to infection with *A. laidlawii* PG8. These proteins are involved in the processes of translation (TufA), photosynthesis (PsaE, PsbP), metabolism (RA, RASIPs, RuBisCO_small, Fdh, AtpA, GAPDH, MDH, MAT, Lip) and defense (TrxM, Glp) of plants. However, the functions of the majority (24 out of 43) of the proteins are not known. The results of analysis of domains in amino acid sequences of some identified hypothetical proteins *in silico* suggest possible functions (Table 2). Proteins Os01g0675100, Os02g0537700, Os07g0694700 may be involved in the maintenance of the redox-status of a cell; OsI_04213, Os01g0654500, Os03g0129300, Os04g0459500, Os12g0420200, Os01g0649100, OsJ_30136, Os03g0169100 — in energy exchange; protein UPP — into integral regulation of cell processes [19,20].

In all, 13 proteins of *O. sativa* L. were responsive toward both adapted and unadapted *A. laidlawii* PG8, but only 3 proteins: MAT (methionine adenosyltransferase — the enzyme that catalyzes the formation of S-adenosylmethionine (AdoMet, an important donor for transmethylation and is also the propylamino donor in polyamine biosynthesis) from methionine and ATP), UPP (carbonic anhydrase — zinc-containing enzyme that catalyze the reversible hydration of carbon dioxide) and hypothetic protein Os01g0654500 showed a similar features of modulating expression (Table 1). Moreover, in the case of plants grown in the medium with unadapted cells of *A. laidlawii* PG8 there was mainly increasing expression level of proteins (29 of 32) (Fig. 3A; Suppl_Fig. 1), while grown with adapted ones — decreasing one (23 of 24 proteins) (Fig. 3B; Suppl_Fig. 1).

To investigate the changes in gene expression at the mRNA level in plants infected with adapted and unadapted *A. laidlawii*, PG8 RT-PCR analysis was performed for four randomly selected differently expressed proteins — TufA, MAT, RASIPs and UPP (Suppl_Fig. 2; Suppl_Table 1). Correlation between the transcriptional profiling data and the data obtained from the proteome analysis was observed in four cases (for TufA and RASIPs in plants infected with adapted cells of the mycoplasma; for MAT in plants infected with adapted and unadapted ones). In other cases a discrepancy between transcriptome and proteome data was observed. Our results confirmed the widely held view that the level of mRNA is not necessarily correlated well with the protein level. Post-translational regulatory events strongly influence the cell protein pattern. The mechanisms of regulating expression for the same proteins of *O. sativa* L. seem to be quite different in plants infecting with adapted to UFE *A. laidlawii* PG8 and unadapted one, which need further exploration.

In plants infected with unadapted mycoplasma cells there was activation of basic cell processes (translation, photosynthesis, energy metabolism, hydrocarbon transport and metabolism) including defensive mechanisms and deactivation of coenzyme metabolism (considering functions of differentially expressed proteins). In plants infected with adapted mycoplasma cells, translation, photosynthesis, hydrocarbon transport and metabolism, energy and coenzyme metabolism were inhibited.

Strong stressors usually inhibit the expression of the majority of proteins [21]. Because we did not detect an invasion of mycoplasma cells into plant tissues, the nature and strength of this stressor remain very interesting. It is very likely that the observed phenomenon is mediated by secreted mycoplasma toxins packed into membrane vesicles involved in protein secretion, intercellular interactions and pathogenesis. It has been reported that membrane vesicles may play significant role in bacterial pathogenicity [22,23]. However, whether these vesicles are involved in the pathogenicity of *A. laidlawii* PG8 to *O. sativa* L. remains to be established.

In the literature, there are data on changes that occur in the proteome of *O. sativa* L. leaves in response toward some bacterial infections [24,25]. However, our work is the first to study the changes in protein expression in *O. sativa* infected by *A. laidlawii* PG8. Changes in the expression of some proteins (TufA (2 isoforms), RA, AtpA, GAPDH (2 isoforms), Glp, MDH (2 isoforms), Os03g0129300 (2 isoforms), Os04g0459500, Os01g0649100, Os07g0694700, OsJ_30136, Os02g0537700, Os01g0675100) in *O. sativa* L. following infection with *A. laidlawii* PG8 and other bacteria were similar. Some proteins (27) were initially revealed in our study as responsive to infection with bacterium. These data suggest a difference between the reaction of *O. sativa* L. toward various bacteria and the mycoplasma cells formed in VCE. In this connection, the functions of the hypothetical proteins UPP, OsI_04213, Os06g0114000, Os01g0654500, Os11g0163100, Os03g0129300, Os04g0459500, Os12g0420200, Os03g0327600, Os01g0649100, Os07g0614500, Os08g0191700, Os02g0133800, Os01g0501800, Os07g0694700, OsJ_30136, Os03g0169100, Os02g0537700, Os07g0500300, Os01g0675100 are of a significant interest from the viewpoint of the proteins of *O. sativa* L. that respond to infection by *A. laidlawii* PG8.

The differences in the responses of *O. sativa* L. toward *A. laidlawii* PG8 grown under optimal and stress conditions may be related to significant differences in virulence of unadapted and adapted to UFE mycoplasma cells, and may be mediated by features of their genome expression and traffic of proteins. Pathologic processes in plants grown in the medium with unadapted and adapted to UFE mycoplasma cells showing the absence of actual invasion of tissues may have been induced by metabolites secreted by the bacterium. Data obtained from TEM and AFM suggest that significant differences existed between the morphology and ultrastructure of cells in control and experimental cultures of *A. laidlawii* PG8 (Figs. 4 and 5). Continuous cultivation of *A. laidlawii* PG8 in media with limited nutrients increased the number of ultramicroforms

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Table 2 – Domain organization of the identified stress-responsive hypothetical proteins of *O. sativa* L.

Spot no.	Protein name	Domain	Description
1	2	3	4
21	Unknown protein (UPP)	Beta_CA_cladeB	Carbonic anhydrase. Carbonic anhydrase are zinc-containing enzyme that catalyze the reversible hydration of carbon dioxide. CAs are ubiquitous enzymes involved in fundamental processes like photosynthesis, respiration, pH homeostasis and ion transport.
22	Hypothetical protein (OsI_04213)	PLN02538	Sulfatase super family. This family includes phosphopentomutase and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase
23	Hypothetical protein (Os06g0114000)	GroEL	Chaperonins are involved in productive folding of proteins.
24	Hypothetical protein (Os06g0114000)	GroEL	Chaperonins are involved in productive folding of proteins.
25	Hypothetical protein (Os01g0654500)	Icd	Isocitrate/isopropylmalate dehydrogenase. Isocitrate dehydrogenase is an important enzyme of carbohydrate metabolism which catalyzes the oxidative decarboxylation of isocitrate into alpha-ketoglutarate. 3-isopropylmalate dehydrogenase catalyzes the third step in the biosynthesis of leucine.
26	Hypothetical protein (Os11g0163100)	ACTIN	Actin. An ubiquitous protein involved in the formation of filaments that are a major component of the cytoskeleton.
27	Hypothetical protein (Os03g0129300)	GapA	Glyceraldehyde-3-phosphate dehydrogenase. GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis.
28	Hypothetical protein (Os03g0129300)	GapA	Glyceraldehyde-3-phosphate dehydrogenase. GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis.
29	Hypothetical protein (Os04g0459500)	GapA	Glyceraldehyde-3-phosphate dehydrogenase. GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis.
30	Hypothetical protein (Os12g0420200)	WcaG	Nucleoside-diphosphate-sugar epimerases. Protein involved in the cell envelope biogenesis, carbohydrate transport and metabolism.
31	Hypothetical protein (Os03g0327600)	not found	
32	Hypothetical protein (Os03g0327600)	not found	
33	Hypothetical protein (Os01g0649100)	RPE	Malate dehydrogenase. MDH is one of the key enzymes in the citric acid cycle, facilitating both the conversion of malate to oxaloacetate and replenishing levels of oxalacetate by reductive carboxylation of pyruvate.
34	Hypothetical protein (Os07g0614500)	EF1B	Elongation factor 1 beta (EF1B) guanine nucleotide exchange domain. EF1B catalyzes the exchange of GDP bound to the G-protein, EF1A, for GTP, an important step in the elongation cycle of the protein biosynthesis.
35	Hypothetical protein (Os08g0191700)	PLN02300	Lactoylglutathione lyase. The principal function of lactoylglutathione lyase is the detoxification of methylglyoxal, that is cytostatic at low concentrations and cytotoxic at millimolar concentrations.
36	Hypothetical protein (Os02g0133800)	PRK03996	Proteasome subunit alpha. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme of nonlysosomal protein degradation in both the cytosol and nucleus.
37	Hypothetical protein (Os01g0501800)	MSP super-family	This family consists of the 33 KDa photosystem II polypeptide from the oxygen evolving complex of plants and cyanobacteria.
38	Hypothetical protein (Os07g0694700)	ascorbate_peroxidase	Ascorbate peroxidases. Ascorbate peroxidases are a subgroup of heme-dependent peroxidases of the plant superfamily that share a heme prosthetic group and catalyze a multistep oxidative reaction involving hydrogen peroxide as the electron acceptor.
39	Hypothetical protein (OsJ_30136)	TIM	Triosephosphate isomerase. Triosephosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate.
40	Hypothetical protein (Os03g0169100)	MDH_glyoxyso-mal_mito-chondrial	Malate dehydrogenase. MDH is one of the key enzymes in the citric acid cycle, facilitating both the conversion of malate to oxaloacetate and replenishing levels of oxalacetate by reductive carboxylation of pyruvate.
41	Hypothetical protein (Os02g0537700)	PRX_Typ2cys	Typical 2-Cys peroxiredoxin subfamily. PRXs are thiol-specific antioxidant proteins, which confer a protective role in cells through its peroxidase activity by reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides.
42	Hypothetical protein (Os07g0500300)	C2_ArfGAP	C-terminal domain ArfGAP. ArfGAP is a GTPase activating protein which regulates the ADP ribosylation factor Arf, a member of the Ras superfamily of GTP-binding proteins.
43	Hypothetical protein (Os01g0675100)	PRX5_like	Peroxiredoxin. PRX5 has been shown to reduce hydrogen peroxide, alkyl hydroperoxides and peroxynitrite.

Note: "Spot no." — a number of a spot on a gel (Fig. 3).

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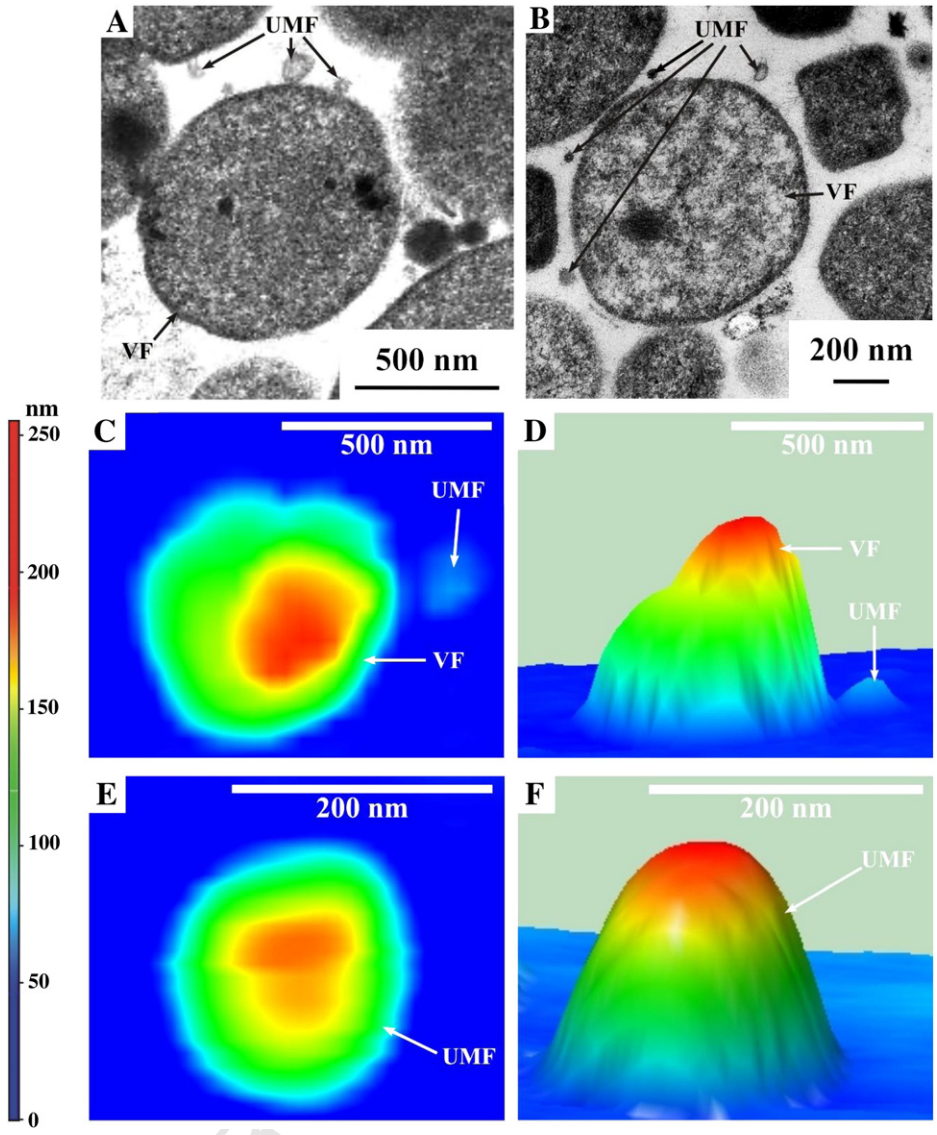

Fig. 4 – Cells and UMFs of the *A. laidlawii* PG8. A and B TEM; C–F AFM (2D — C, E; 3D — D, F). VF — vegetative forms, UMF — ultramicroforms.

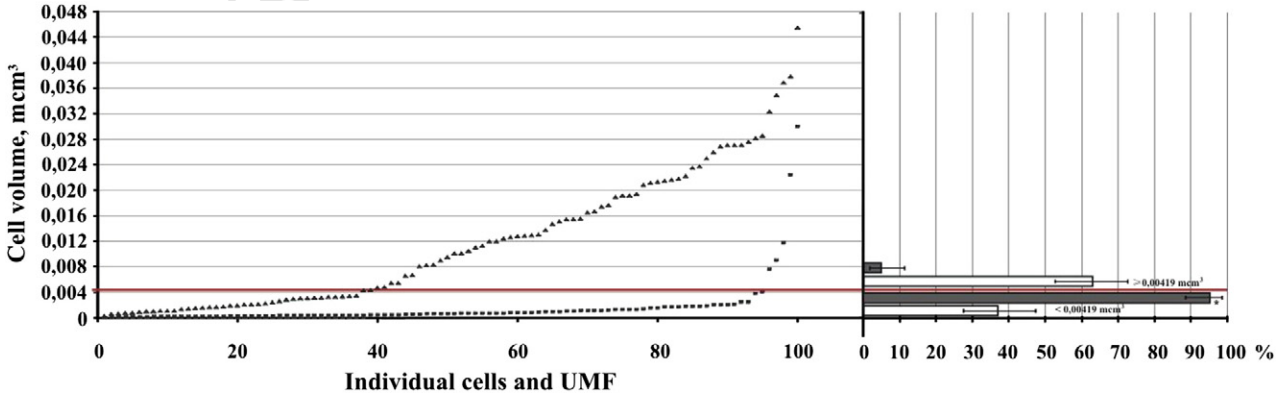

Fig. 5 – Proportion of typical cells and UMF in *A. laidlawii* PG8 culture grown under optimal (▲) and stress (■) conditions. Individual cells and UMF were sorted from the smallest to the largest and graphed accordingly. Each point represents an individual cell or UMF. * — $p < 0.025$.

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(Fig. 5). These are spherical membrane-bound nanostructures whose sizes (d<0.2 m cm; V<0.00419 m cm^3) were less than those of typical mycoplasmas and of minimal cell that is able to undergo autonomic replication [26,27]. According to the sizes, morphology and ultrastructure of the majority of these ultramicroforms, they were similar for membrane vesicles that are produced by bacterial cells, and mediate protein secretion, intercellular interactions and pathogenesis [22,23].

We used two-dimensional gel electrophoresis with mass spectrometry analysis to study the expression patterns of proteins of *A. laidlawii* PG8 cells grown under optimal and stress conditons. 2D-DIGE of proteins from mycoplasma cells visualized about 300 protein spots. Theoretically, the proteome of *A. laidlawii* PG8 comprises about 1380 proteins. Most of them (~60% for this mycoplasma) are membrane proteins. The limitation of 2-DE for membrane proteins is well-known. 2-DE cannot properly resolve high molecular weight, very basic or hydrophobic proteins. Considering low-copy proteins and above-mentioned, an ability to visualize 300 visualized protein spots appears to be an acceptable resolution.

The polypeptide spectra of mycoplasmas that were unadapted and adapted to UFE were quite different (Fig. 6, Suppl_Fig. 3). The differences were related to changes in the expression of proteins (including isoforms) and their shifting to low pI. From 2D-DIGE of proteins from cells of control and experimental *A. laidlawii* PG8 cultures, 82 differentially expressed proteins were registered and identified (Fig. 6, Suppl_Fig. 3, Table 3). Accordingly to COG functional categories, the proteins are involved in replication, repair, recombination (ParE, GyrB, LigA, UvrA, Nfo), transcription (RpoB, Mar, Tex, LacI, GreA), translation (TufB, EF-G, Tsf, RpsA, RpsF, PNPase, GlnRS, ProRS, GlyRS, SerRS, PheRS, AspRS, HisRS), energy production (AAD, PdhA, PdhB, PdhC, PdhD, AckA, AtpD), transport and metabolism of carbohydrates (Kba, TpiA, Pgk, Pgm, AmyA, ACL_0205, Hpr2, ACL_0240), nucleotides (Prs, Rnr, Cmpk, DeoA) and amino acids (PepF, ArgE, AmpT, GlyA)

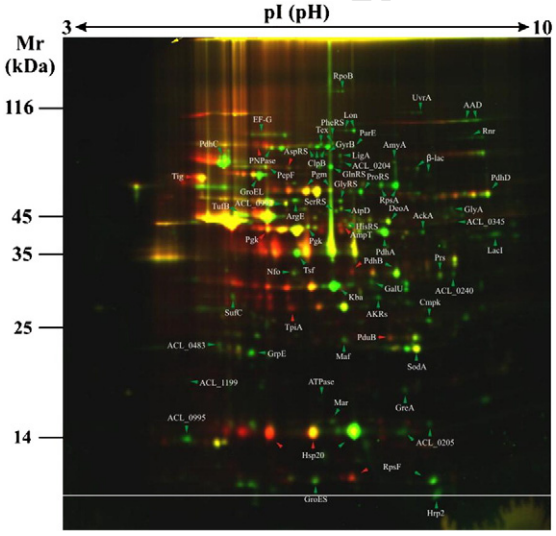

Fig. 6 – 2D-DIGE analyses of proteins from adapted (●) to UFE *A. laidlawii* PG8 cells and unadapted (●) ones. Arrows indicate differentially expressed mycoplasma proteins.

as well as secondary metabolites biosynthesis (PduB), signal transduction mechanisms (ACL_1199, ACL_0204) and virulence (Table 3). Some of these proteins were previously detected as differently expressed in various bacteria at starvation, for example, Pgm, PNPase, EF-G, EF-Tu, TpiA in *Vibrio cholerae* O1 [28], EF-G, EF-Ts, GyrB, SodA, Pgm, PdhB, GroEL, RpsF, TpiA, PdhA in *Bacillus subtilis* 168 [21], PdhB, Pgk, Pgm, TpiA in *Bacillus licheniformis* DSM 13 [29]. The majority of differentially expressed proteins (86.5%) were down-regulated. Considering the functions of those proteins, it is possible to conclude that stress conditions (starvation) inhibits in *A. laidlawii* PG8 cells basic cell processes related to replication, recombination, reparation, transcription, translation, post-translation modification, protein metabolism, chaperones, energy production, transport and metabolism of carbohydrates, amino acids, nucleotides and inorganic ions. This is largely in agreement with data of analogous research for other microorganisms. Changes in the expression level of proteins during the starvation of bacteria were considered to promote survival, utilization of alternative energy sources, entrance into a hypometabolic state and a change in virulence. This might be in the case of *A. laidlawii* PG8. Earlier [1,5] we reported that starvation leads *A. laidlawii* PG8 to dormancy. However, the role of the proteins that we identified as being differently expressed in *A. laidlawii* PG8 cells during the process of adaptation to UFE remains to be studied.

From the viewpoint of adaptation to stress conditions, stress-induced proteins, whose expression level is increased at starvation in bacteria, seem interesting. The expression level of some proteins (RpsF, PNPase, AspRS, Hsp20, trigger factor, PdhB, Kba, Pgk, AmpT, PduB) increased in adapted to UFE cells. This was accompanied by a significant increase of the relative abundance of PNPase, considered to be a global regulator of gene expression and of virulence in phytopathogenic bacteria [30]. That is especially interesting from the viewpoint of the participation of this protein in regulation of adaptation of *A. laidlawii* PG8 toward stressors.

The expression level of most proteins (71) of *A. laidlawii* PG8 decreased in UFE. Specifically, 31 proteins identified in the control culture (including five involved in virulence) were not observed following UFE (Table 3). Among the proteins, there are 11 that showed increased expression level in the short term following the onset of oxidative stress and cold shock [31], but a decreased level during culture at normal refrigeration temperatures (our unpublished data).

To investigate the changes in gene expression at the mRNA level in adapted and unadapted *A. laidlawii* PG8, RT-PCR analysis was performed for six randomly selected identified up- and down-regulated proteins – RpoB, AckA, β-lac, TufB, PNPase and PdhA (Suppl_Fig. 4, Suppl_Table 2). In cases of PNPase, PdhA we observed a discrepancy between the transcriptional profiling and the data obtained from the proteome analysis. In other cases, the transcripts of down-regulated proteins were also down-regulated, but (except for TufB) were down-regulated to a greater extent. These observations indicate that post-transcriptional regulatory events strongly influence the protein pattern of UFE adapted *A. laidlawii* PG8 cells.

Among the proteins of *A. laidlawii* PG8 that displayed the greatest change in expression level were those which are also known to be secreted via membrane vesicles (MVs) in some

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Table 3 – Differentially expressed proteins of *A. laidlawii* PG8 cells cultivated under optimal and stress conditions.

Spot no.	COG[1]	Protein name	Score	SC (%)[2]	Number of mass values searched	Number of mass values matched	Number of mass values no matched	NCBI accession number[3]	Theoretical Mr (Da)/pI[4]	Experimental Mr (Da)/pI[5]	Fold change[6]
1	2	3	4	5	6	7	8	9	10	11	12
1	L	DNA topoisomerase IV, subunit B (ParE)	302	25	32	27	5	gi|162447245	72,936/5.78	73,589/5.77	K
2	L	DNA gyrase, subunit B (GyrB)	142	19	15	13	2	gi|162446893	71,171/5.61	66,037/5.69	K
3	L	DNA ligase (LigA)	81	14	14	8	6	gi|162448184	73,963/5.67	67,816/5.60	−9.506 ± 1.263
4	L	Excinuclease ABC, subunit A (UvrA)	61	12	25	10	15	gi|162448183	105,008/6.21	101,919/6.28	K
5	L	Endodeoxyribonuclease IV (Nfo)	86	23	12	8	4	gi|162447345	33,100/5.40	33,990/5.39	−3.378 ± 0.775
6	D	Septum formation protein (Maf)	116	36	11	8	3	gi|162448218	21,102/5.43	23,150/5.73	−1.705 ± 0.240
7*	K	DNA-directed RNA polymerase, subunit beta (RpoB)	302	25	39	33	6	gi|162447041	141,351/5.71	118,623/5.70	K
8	K	MarR family transcriptional regulator (Mar)	140	66	18	10	8	gi|162447905	14,582/4.96	15,532/5.64	−2.894 ± 0.232
9	K	Tex protein-related transcription accessory protein (Tex)	272	43	42	27	15	gi|162448175	81,687/5.67	76,981/5.58	K
10	K	Tex protein-related transcription accessory protein (Tex)	81	10	9	7	2	gi|162448175	81,616/5.67	76,210/5.65	K
11	K	LacI family transcription regulator (LacI)	99	21	15	8	7	gi|162448029	36,678/6.86	40,521/6.85	−12.004 ± 2.799
12	K	Transcription elongation factor (GreA)	231	65	13	12	1	gi|162447758	17,715/5.99	18,768/6.22	K
13*	J	Translation elongation factor Tu (TufB)	261	68	51	26	25	gi|162447058	42,824/5.21	45,565/5.04	−3.610 ± 0.688
14*	J	Translation elongation factor G (EF-G)	406	50	39	35	4	gi|162447164	76,287/5.29	86,867/5.10	−4.202 ± 0.283
15	J	Translation elongation factor Ts (Tsf)	226	53	20	15	5	gi|162447999	32,085/5.42	37,418/5.41	−3.057 ± 0.139
16	J	Small subunit ribosomal protein S1 (RpsA)	269	34	19	19	0	gi|162447732	54,069/6.07	55,773/6.04	−5.933 ± 0.825
17	J	Small subunit ribosomal protein S1 (RpsA)	216	27	19	18	1	gi|162447732	54,069/6.07	55,714/6.17	−41.857 ± 2.250
18	J	Small subunit ribosomal protein S6 (RpsF)	76	35	7	5	2	gi|162447063	10,777/6.60	10,600/5.77	3.949 ± 0.353
19	J	Small subunit ribosomal protein S6 (RpsF)	76	35	6	4	2	gi|162447063	10,777/6.60	10,680/6.36	−4.390 ± 0.303
20	J	Polyribonucleotide nucleotidyltransferase (PNPase)	72	10	19	9	10	gi|162447666	78,617/5.33	77,644/5.14	5.502 ± 0.327
21	J	Polyribonucleotide nucleotidyltransferase (PNPase)	347	48	55	36	19	gi|162447666	78,617/5.33	74,907/5.30	−5.024 ± 0.391
22	J	Glutaminyl-tRNA synthetase (GlnRS)	291	44	36	29	7	gi|162448188	62,840/5.58	62,090/5.63	K
23*	J	Prolyl-tRNA synthetase (ProRS)	245	40	30	23	7	gi|162447162	54,218/5.82	53,515/5.83	K
24*	J	Glycyl-tRNA synthetase. class II (GlyRS)	119	21	12	9	3	gi|162447800	53,116/5.60	49,109/5.69	−6.077 ± 0.691

25	J	Seryl-tRNA synthetase (SerRS)	81	16	10	7	3	gi|162446896	48,233/5.65	44,920/5.66	−6.129 ± 1.534
26	J	Phenylalanyl-tRNA synthetase (PheRS)	292	38	50	31	19	gi|162447120	87,473/5.64	81,359/5.63	K
27	J	Aspartyl-tRNA synthetase (AspRS)	411	59	49	39	10	gi|162447681	65,376/5.37	62,436/5.35	1.918 ± 0.481
28	J	Histidyl-tRNA synthetase (HisRS)	296	60	34	25	9	gi|162447682	48,211/5.69	43,281/5.73	−1.714 ± 0.135
29	O	Chaperonin GroEL (GroEL)	474	70	38	37	1	gi|162448090	58,231/5.17	60,462/5.21	−4.865 ± 0.181
30	O	Molecular chaperone, heat shock protein Hsp20 (Hsp20)	136	32	3	2	1	gi|162447286	16,044/5.69	14,857/5.22	3.913 ± 0.045
31	O	Molecular chaperone, heat shock protein Hsp20 (Hsp20)	126	70	13	9	4	gi|162447286	16,044/5.69	14,635/5.51	1.513 ± 0.021
32	O	Molecular chaperone, heat shock protein Hsp20 (Hsp20)	63	29	18	6	12	gi|162447286	16,044/5.69	14,806/5.78	−9.030 ± 0.323
33	O	Chaperonin GroES/Hsp10 (GroES)	108	59	7	6	1	gi|162448091	9541/5.54	10,218/5.53	−2.189 ± 0.1242
34	O	Molecular chaperone GrpE (GrpE)	110	41	15	8	7	gi|162447411	22,000/5.14	22,060/5.11	−6.837 ± 0.734
35	O	Trigger factor. FKBP-type peptidyl-prolyl cis-trans isomerase (Trigger factor)	360	65	43	30	13	gi|162447397	48,236/4.75	59,356/4.72	1.543 ± 0.124
36	O	ATP-dependent Clp protease ATP-binding subunit (ClpB)	111	23	26	12	14	gi|162447977	80,199/5.58	74,039/5.61	K
37	O	ATP-dependent Clp protease ATP-binding subunit (ClpB)	183	27	30	18	12	gi|162447977	80,199/5.58	74,227/5.55	K
38	O	ATP-dependent Clp protease ATP-binding subunit (ClpB)	381	55	57	44	13	gi|162447977	80,057/5.58	74,934/5.50	K
39	O	Serine protease Lon. ATP-dependent (Lon)	324	39	33	31	2	gi|162447398	86,710/5.76	86,196/5.73	K
40	O	Serine protease Lon. ATP-dependent (Lon)	397	47	51	43	8	gi|162447398	86,639/5.76	86,053/5.78	K
41	O	SUF system FeS cluster assembly protein (SufC)	226	67	27	20	7	gi|162448060	28,419/4.97	29,810/4.99	K
42	O	Peptidase U32 family protein (ACL_0345)	317	58	27	27	3	gi|162447211	44,890/6.48	43,305/6.52	K
43	C	Bifunctional protein: aldehyde and alcohol dehydrogenase (AAD)	245	27	28	23	5	gi|162447047	95,048/6.80	95,117/6.61	−1.571 ± 0.117
44	C	Bifunctional protein: aldehyde and alcohol dehydrogenase (AAD)	242	27	30	25	5	gi|162447047	95,048/6.80	96,277/6.72	−2.122 ± 0.335
45*	C	Pyruvate dehydrogenase E1 component, alpha subunit (PdhA)	134	26	25	13	12	gi|162448151	40,843/5.82	41,405/6.07	−9.882 ± 1.507
46*	C	Pyruvate dehydrogenase E1 component, beta subunit (PdhB)	123	32	16	11	5	gi|162448150	35,660/6.04	34,209/5.77	5.500 ± 1.109
47*	C	Pyruvate dehydrogenase E1 component, beta subunit (PdhB)	173	47	24	16	8	gi|162448150	35,660/6.04	34,083/6.18	−3.213 ± 0.268
48	C	Dihydrolipoamide acetyltransferase (PdhC)	65	12	15	6	9	gi|162448149	57,225/5.00	65,659/4.92	−11.600 ± 1.738
49	C	Dihydrolipoamide dehydrogenase (PdhD)	186	43	30	18	12	gi|162448148	49,150/6.72	51,763/6.81	−1.620 ± 0.091

(continued on next page)

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Table 3 *(continued)*

Spot no.	COG[1]	Protein name	Score	SC (%)[2]	Number of mass values searched	Number of mass values matched	Number of mass values no matched	NCBI accession number[3]	Theoretical Mr (Da)/pI[4]	Experimental Mr (Da)/pI[5]	Fold change[6]
1	2	3	4	5	6	7	8	9	10	11	12
50*	C	Acetate kinase (AckA)	86	22	13	7	6	gi|162447358	42,906/6.47	41,602/6.29	K
51*	C	F-type H+−transporting ATPase beta chain (AtpD)	182	36	19	16	3	gi|162447836	50,427/5.66	46,142/5.70	−2.254 ± 0.291
52	G	Alpha-amylase (AmyA)	319	39	39	31	8	gi|162447517	71,136/6.04	64,572/6.17	−7.427 ± 2.973
53	G	Ketose bisphosphate aldolase. class-II (Kba)	246	69	33	24	9	gi|162448230	30,391/5.47	31,511/5.22	2.549 ± 1.043
54	G	Triosephosphate isomerase (TpiA)	81	29	8	6	2	gi|162448049	27,785/5.64	27,374/5.72	−3.776 ± 0.408
55	G	Phosphoglycerate kinase (Pgk)	290	62	29	23	6	gi|162448052	42,741/5.43	42,382/5.17	2.174 ± 0.019
56	G	Phosphoglycerate kinase (Pgk)	310	63	33	25	8	gi|162448052	42,741/5.43	41,577/5.41	−2.247 ± 0.411
57	G	Phosphoglyceromutase (Pgm)	323	62	30	26	4	gi|162447265	55,799/5.58	52,777/5.55	−1.786 ± 0.264
58	G	Phosphoglyceromutase (Pgm)	208	44	24	17	7	gi|162447265	55,942/5.58	53,217/5.63	−1.501 ± 0.145
59	G	RpiB/LacA/LacB family sugar-phosphate isomerase (ACL_0205)	88	34	9	5	4	gi|162447075	16,033/6.23	15,117/6.22	K
60	G	RpiB/LacA/LacB family sugar-phosphate isomerase (ACL_0205)	213	62	14	11	3	gi|162447075	16,033/6.23	15,691/6.32	K
61	G	Phosphotransferase system, phosphocarrier protein (Hpr2)	105	61	7	5	2	gi|162448050	8,546/7.98	9,204/6.41	K

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62	G	Hypothetical protein (ACL_0240)	148	39	17	11	6	gi|162447110	35,467/6.42	33,397/6.52	K
63	E	Glycine hydroxymethyltransferase (GlyA)	175	24	15	13	2	gi|162446933	45,296/7.18	46,474/6.50	K
64	E	Oligoendopeptidase F (PepF)	243	34	30	23	7	gi|162446989	65,591/5.25	63,708/5.20	−2.948±1.631
65	E	Acetylornithine deacetylase (ArgE)	183	35	19	14	5	gi|162447552	51,447/5.40	49,995/5.40	−5.941±0.374
66	E	Aminopeptidase T (AmpT)	83	17	19	8	11	gi|162447107	46,251/5.79	42,365/5.69	3.874±1.172
67*	E/F	Ribose-phosphate pyrophosphokinase (Prs)	80	22	12	7	5	gi|162446905	34,894/6.33	33,313/6.39	−1.733±0.130
68	F	Ribonucleoside-triphosphate reductase (Rnr)	213	28	20	18	2	gi|162448132	86,417/6.85	81,329/6.68	K
69	F	Cytidylate kinase (Cmpk)	221	55	15	14	1	gi|162447733	25,105/6.24	25,588/6.32	−5.367±0.490
70	F	Thymidine phosphorylase (DeoA)	281	56	32	25	7	gi|162447543	46,563/5.98	43,529/6.11	−2.273±0.138
71	P	Fe/Mn family superoxide dismutase (SodA)	110	59	26	8	18	gi|162447168	22,826/6.18	22,679/6.26	−1.792±0.114
72	Q	Propanediol utilization protein (PduB)	112	41	11	7	4	gi|162446900	21,299/6.17	23,798/6.12	2.272±0.377
73	M	UDP glucose pyrophosphorylase (GalU)	54	12	3	3	0	gi|162447697	31,983/5.82	32,507/5.93	K
74	T	Hypothetical protein (ACL_1199)	52	24	15	5	10	gi|162448042	17,066/4.59	19,091/4.63	K
75	T	Translational GTPase, TypA/BipA type (ACL_0204)	201	26	25	18	7	gi|162447074	67,777/5.62	63,881/5.70	K
76*	R	Metallo-beta-lactamase superfamily protein (β-lac)	273	39	18	18	0	gi|162447175	62,291/6.27	62,109/6.26	−9.119±2.798
77*	R	Metallo-beta-lactamase superfamily protein (β-lac)	304	47	31	26	5	gi|162447690	64,018/6.49	58,677/6.31	K
78	R	Aldo/keto reductase family oxidoreductase (AKRs)	105	31	10	7	3	gi|162447625	30,785/5.82	29,449/6.00	K
79	R	ATPase (ATPase)	121	45	7	6	1	gi|162448193	16,713/5.35	17,487/5.56	K
80	R	Zn-dependent protease (ACL_0992)	255	47	32	24	8	gi|162447846	50,277/5.25	48,998/5.33	−1.697±0.188
81	-	hypothetical protein (ACL_0483)	106	37	15	7	8	gi|162447346	24,365/4.98	22,588/4.86	−4.785±2.815
82	-	hypothetical protein (ACL_0995)	84	36	9	6	3	gi|162447849	14,217/4.60	13,986/4.62	K

Note: [1]The functional categories were decided according to COG: L — replication, recombination and repair, D — cell cycle control, mitosis and meiosis, K — transcription, J — translation, O — posttranslational modification, protein turnover, chaperones, C — energy production and conversion, G — transport and metabolism of carbohydrates, E — amino acid transport and metabolism, F — nucleotide transport and metabolism, P — inorganic ion transport and metabolism, Q — secondary metabolites biosynthesis, transport and catabolism, M — cell wall/membrane biogenesis, T — signal transduction mechanisms, R — general function prediction only. «-» — not in COGs; [2]sequence coverage; [3]identification number of a protein in NCBI database; [4]theoretical molecular weight and pI; [5]experimental molecular weight and pI; [6]mean±standart deviation. K — protein is presented only in unadapted to UFE *A. laidlawii* PG8 cells. * — proteins associated with secretion in membrane vesicles in gram-positive bacteria [25]; — virulence proteins in various bacteria.

gram-positive bacteria [25], the nearest relatives of mycoplasmas (Table 3). It is probable that changes in virulence, expression of proteins and the intensive production of membrane vesicles in *A. laidlawii* PG8 culture during starvation are interrelated processes. Obviously, proteome analysis of membrane vesicles secreted by unadapted and adapted to UFE *A. laidlawii* PG8 cells may help us to understand the adaptation of this bacterium to UFE and its phytopathogenicity.

4. Conclusions

We reported previously [5] that *A. laidlawii* PG8, a ubiquitous mycoplasma found in soil, compost, waste waters, and in insects, may also cause phytoplasmoses in various plants. This pathogen is important in agriculture [3]. However, data on phytopathogenicity of *A. laidlawii* PG8 toward *O. sativa* L. are absent in the literature. Therefore, we performed the present work.

For the first time, a proteomic approach was carried out to identify leaf proteins in *O. sativa* L. that are involved in the response of rice to infection with *A. laidlawii* PG8. Using 2D-DIGE and MS, 43 proteins of *O. sativa* L. that were differentially expressed in leaves of plants cultivated in media with *A. laidlawii* PG8 were identified. The majority of the identified proteins that were differentially expressed in plants while infecting with mycoplasma was initially detected in our study as responsive ones toward infection with bacterium. A difference in the response of *O. sativa* L. toward unstressed mycoplasma cells and those formed under optimal and stress conditions was found. So, 13 proteins of *O. sativa* L. were responsive toward both adapted and unadapted *A. laidlawii* PG8 but only 3 proteins (MAT, UPP, Os01g0654500) showed similar features of the modulating expression. The differences in the responses of *O. sativa* L. toward *A. laidlawii* PG8 grown under optimal and stress conditions were related to virulence of unadapted and adapted to UFE mycoplasma cells. Adapted cells of the mycoplasma differed from unadapted ones by displaying a significant increase in the expression of PNPase. This enzyme is a global regulator of virulence in phytopathogenic bacteria. Adapted cells displayed a decreased expression level of 40 proteins (including 14 involved in bacterial virulence) and expression of 31 proteins (including 5 involved in virulence) was not detected. Adapted mycoplasma cells also caused changes in ultrastructure and protein expression in rice leaves, and unlike unadapted cells, did not invade leaf tissues through the root system. Data from PCR-analysis showed that DNA from adapted *A. laidlawii* PG8 was absent in rice tissues (roots and leaves), and this was confirmed by TEM data. These data allow us to suggest that adapted *A. laidlawii* PG8 cells, unlike unadapted cells, do not invade *O. sativa* L. tissue through the root system. PCR-analysis of plant roots confirmed the absence of *A. laidlawii* PG8 (adapted and unadapted) DNA in the corresponding tissues (data not shown). This allows us to conclude that the invasion of the mycoplasma into plant tissues (at least, of adapted to UFE) occurs at earlier (to our analysis of the samples) time period. Clarification of this issue is of special interest and needs further investigation.

Meanwhile, despite the absence of invasion of plants with adapted *A. laidlawii* PG8 cells, alterations occurred in the ultrastructure of plants grown in media with adapted cells. This proves that adapted cells of the mycoplasma are able to cause pathology in plants, i.e. cells of *A. laidawii* PG8 are virulent. In this case, mycoplasma cells may be located at the surface of root hairs of *O. sativa* L., and affect their negative actions via metabolites secreted by bacterial cells, for example, via membrane vesicles. In the adapted to UFE *A. laidlawii* PG8 culture an increasing amount of spherical membrane-bound nanostructures were found. Their sizes, morphology and ultrastructure were similar to membrane vesicles mediating the secretion of proteins and biologically active metabolites by bacteria, which are involved in intercellular interactions and pathogenesis [32]. In this connection, comparative proteomics of membrane vesicles, secreted by *A. laidlawii* PG8 cells grown under optimal and stress conditions, will be of a special interest for elucidating adaptation mechanisms of the bacterium and its phytopathogenicity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2011.07.016.

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