Iran. J. Biotechnol. July 2021;19(3): e2722 DOI: 10.30498/ijb.2021.223625.2722

Research Article

Proteome Changes in Stem Tissues of Sunflower Lines Inoculated with Culture Filtrate of *Sclerotinia sclerotiorum*

Maryam Monazzah[1], Sattar Tahmasebi Enferadi[1,*]; Zohreh Rabiei[1], Maria De Nobili[2]

[1]Department of Plant Molecular Biotechnology, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
[2]Department of Agriculture and Environmental Sciences, University of Udine, Italy.

[*]*Corresponding author:* Sattar Tahmasebi Enferadi, Department of Plant Molecular Biotechnology, Institute of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran. Tel/Fax: +98-2144787301, E-mail: tahmasebi@nigeb.ac.ir

Background: *Sclerotinia sclerotiorum* (Lib.) de Bary cause a deleterious disease on sunflower plants. Oxalic acid is the main pathogenicity factor of *S. sclerotiorum*. Two dimensional gel electrophoresis and mass spectrometry have been used in several studies to investigate molecular changes that occur in the plants in response to *S. sclerotiorum* infection. Comparing responses of resistant and susceptible lines upon pathogen infection provided novel information regarding defense mechanisms against this necrotrophic pathogen.

Objectives: The present study reports proteome changes of partially resistant and susceptible sunflower lines under pathogen's culture filtrate treatment, resulting in the characterization of up- and down- regulated proteins.

Material and Methods: Sunflower partially resistant and susceptible lines with two true leaves were exposed to fungus culture filtrate. The stems of treated and untreated plants were sampled at 24, 48 and 72 hours after treatment for two-dimensional electrophoresis. Twenty spots showed more than 1.5-fold change in abundance were subjected to MALDI/TOF-TOF MS for further analysis.

Results: The identified proteins were categorized into several classes including carbohydrate and energy metabolism (25%), cellular metabolic process (15%), stress response (15%), plant cell wall biogenesis (10%), photosynthesis (10%), protein metabolism (10%), unknown function (10%) and redox homeostasis (5%).

Conclusions: Our proteomic investigation demonstrates an increase in the expression of proteins only in partially resistant line, such as proteins involved in carbohydrate metabolism and plant defense responses (malate dehydrogenase and peroxidase), metabolic process (adenosine kinase), regulating cell redox homeostasis (disulfide isomerase) and lignin biosynthetic process (laccase). Moreover, the expression of pyrroline-5-carboxylate reductase, involved in proline biosynthesis, was significantly changed in both sunflower lines in response to pathogen culture filtrate. Proteins which were only up-regulated in the partially resistant lines might have a significant role in mediating the defense against Sclerotinia and could be considered for enhancing resistance against this devastating pathogen.

Keywords: Biotic stress; *Sclerotinia*; Sunflower; Tolerance; 2-DE

1. Background

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic pathogen which infect a wide range of plants, including many important oilseed crops such as sunflower, canola and soybean (1).

Oxalic acid (OA; $H_2C_2O_4$) is a non-specific toxin of many necrotrophic pathogens that is definitely accepted as the main virulence factor of *S. sclerotiorum* (2). In addition, Xiao *et al.* (3) showed that *S. sclerotiorum* secretes a novel protein elicitor (Ss-Caf1), which play a major role in induction of PCD and sclerotia formation in plant tissues. Besides OA, fumaric

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acid accumulation has been associated with disease development in plant hosts (4). OA play diverse roles during infection including direct toxicity to plant tissues (5), raising the activities of cell wall degrading enzymes (6), promoting endo-polygalacturonase activity (7), enhancing proteinase activity (8), inhibiting the activity of 0-diphenol oxidase (9), removing accumulated toxic calcium ions (10), suppressing production of reactive oxygen species (ROS) in the early stages of infection, and then inducing oxidative burst and programmed cell death (PCD) in plant tissues (11, 12). In addition to OA, *S. sclerotiorum* secretes cell wall degrading enzymes (CWDEs), which can promote the development and penetration of pathogen to host cell walls (13).

Genomic tools provide valuable information about gene expression changes in host-pathogen interactions. However, proteomics-based investigation of such interactions is also important to identify proteins that are pathogen responsive, because transcript level often correlated poorly with protein abundance (14). Two dimensional gel electrophoresis and mass spectrometry are considered powerful tools for understanding biochemical and physiological responses of host to biotic stresses (14).

It is important to identify host proteins involved in defense mechanisms by comparing responses of resistant and susceptible lines that are infected by the pathogen. Several studies (15-20) have investigated molecular changes that occur in the host in response to either OA or *S. sclerotiorum* treatment. Liang *et al.* (15, 16) investigated the proteome changes in *Brassica napus* L. seedlings during treatment with OA and *S. sclerotiorum*. They found that OA decreases the abundance of proteins involved in photosynthesis, and negatively affect host photosynthetic capacity. They also reported that proteins involved in ethylene biosynthesis, such as methionine adenosyltransferase, and JA-signaling, such as JA-responsive proteins were induced in *B. napus* genotype upon pathogen challenge. Wen *et al.* (17) and Garg *et al.* (18) compared protein profile of resistant and susceptible lines of *B. napus* in cotyledon and adult plants during infection with *S. sclerotiorum.* Some proteins involved in defense responses such as a glycine rich protein, a trypsin inhibitor protein, heat shock proteins were only upregulated in the resistant line of the adult plants. These proteins play important roles in scavenging of ROS and defense response, which may confer resistance against *S. sclerotiorum.* Garg *et al.* (2014) showed proteins involved in primary metabolic pathways, antioxidant defense, ethylene biosynthesis and protein folding were specifically increased in resistant genotype, which may regulate defense responses against *S. sclerotiorum.* There are many examples exist in literature that used proteome-based studies to find novel information regarding cellular processes occurring in plant responses to biotic and abiotic stresses that need to be continued in the future. There are differences in defense mechanisms within plant species, thus, interactions in each pathosystem should be studied in details (16, 21). Most proteome studies have already been applied to investigate *Brassica napus- S. sclerotiorum* interaction.

2. Objectives

In the present study, two dimensional gel electrophoresis and tandem mass spectrophotometry have been used to investigate the changes in the stem proteome of susceptible and partially resistant lines of *Helianthus annuus* treated by culture filtrate of *S. sclerotiorum*. The roles of these proteins are discussed in this paper. It was suggested that overexpression or downregulation of the potential proteins may be taken for plant-breeding programs (22).

3. Martial and Methods

3.1. Preparation of S. sclerotiorum Culture Filtrate

Sclerotia of *S. sclerotiorum* were collected directly from naturally infected sunflower fields (Cultivar Farokh) located in Alborz province. They were cultured on potato dextrose agar (PDA) and incubated at 28 °C. Mycelial plug (1 cm diameter) of *S. sclerotiorum* were cut with a sterile scalpel and transferred to liquid Czapek-Dox medium and incubated at 28 °C for one month to have all metabolites of the pathogen in the medium. Then, the mycelia were carefully separated from the liquid medium by a sterile muslin cloth and medium was filtered through the 0.22 µm sterile filters (25). The control medium was liquid Czapek-Dox medium inculcated by a PDA without mycelia. The content of OA as the main component in the liquid medium was determined by high performance liquid chromatography (HPLC). HPLC analysis was carried out using Agilent ZORBAX Eclipse XDB-C18 column (4.6 × 250 mm) with UV-detector set at 240 nm. In order to determine OA concentration in the liquid broth, standard curves generated with known concentrations of OA (**supplementary file 1**).

3.2. Plant Material and Treatment

Screening tests have been done at Udine University, Italy to evaluate the susceptibility of partially resistant (AC 4122) and susceptible HA 89 (Reg. no. GS-39, PI 642062) sunflower lines to *S. sclerotiorum* (24). AC4122 and HA 89 are maintainer inbred lines, developed at Udine University from an Italian open pollinated population (ALA) (25). Sunflower seeds were germinated on ½ MS medium enriched with vitamins (thiamine, nicotinic acid and pyridoxine). The experiment was established in a completely random design with three replicates. When the 14-day old sunflower seedlings were at the first true leaf, the stems were slightly scratched by a sterilized tip (16) and then treated with 10 mL pathogen culture filtrate (supplementary file 1). The treated tissue stems were collected 24, 48 and 72 hours post treatment and store at -70 °C until use. The stems were treated by Czapek-Dox media as control (26).

3.3. Total Protein Extraction

Briefly, 0.5 gram of *H. annuus* stems were pooled and ground to a fine powder using liquid nitrogen. Then, the powdered tissue was transferred to 2 mL microtubes. Cold 10% TCA/acetone containing 0.07% DTT (w/v) and 1mM PMSF was added to the samples and stored overnight at -20 °C to promote protein precipitation. Then, they were centrifuged at 16000 g for 20 minutes at 4 °C. The supernatant carefully was removed and the protein pellet was resuspended four times with 1 mL cold acetone containing 0.07% (w/v) DTT. The pellet was completely dried by Speed–Vac and the proteins were solubilized in 400 µL lysis buffer (8 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS and ampholite 3-10). The microtubes were vigorously vortexed for 30 min at room temperature and then centrifuged (16000 g for 30 min). The supernatant was collected and store at -70 °C until use (16). The Bradford protein assay method was used to measure protein concentration in samples.

3.4. Two-dimensional Electrophoresis

IPG gel strips (7 cm length; pH 3-10 NL; GE Healthcare) were passively rehydrated with 125 µL rehydration buffer (8 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS and 0.2% ampholyte pH 3–10) containing 100 µg extracted proteins for 16 hours. Then, isoelectric focusing was performed on an Amersham GE Healthcare Ettan IPGphor 3 at 20 °C for 56 kVh. Before second dimension, IPG strips were equilibrated for 20 min in 3 mL equilibration buffer containing 6 M urea, 2% SDS, 0.05 M Tris-HCl, pH 8.8, 30% glycerol containing 1% DTT and then incubated for another 20 min in the same buffer containing 4% iodoacetamide instead of DTT. The equilibrated strips were loaded on top of 12% polyacrylamide gels in a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA). Finally, the gels were stained with Coomassie Brilliant Blue R250 (CBB-R250) for 4 hours and then destained with 40% HPLC-grade methanol and 10% acetic acid until protein spots were clearly visible (16).

3.5. Image Analysis

Images of CBB-stained 2-DE gels were recorded using an Image scanner III (GE Healthcare, USA). Analysis of the 2–D gels was performed by ImageMaster 2D platinium version 6.02 (GE Healthcare, sweden). Protein spots were detected using the automated detection tool of software. In addition, all the apparent artifacts were removed and any missed spots were manually added. A matchset was made including all images from each replicate for both treated and control samples. Finally, only spots present in all the replicates of each treatment were considered for further analysis. The abundance of each protein spot was measured as the volume percentage of the spot (% vol.) that were determined by dividing the individual spot volumes by the total volume of all spots observed in the gel. The Student's t-test comparisons between treated and control samples were performed by using these values of % vol. for both partially resistant and susceptible lines at each specific time point (partially resistant vs its control and susceptible vs its control at 24, 48 and 72 h post treatment). Finally, Spots with at least a 1.5-fold change (up or down regulation) and significant at the $p \leq 0.05$ level were considered for identification. Then, these protein spots were excised from gels and they were analyzed by University of York, Department of Biology, UK for identification by Mass spectrometer (MALDI-TOF/TOF). Expression ratios or fold changes for each spot in both lines were determined from the spot vol. % data with respect to their control at each time points (16, 17).

3.6. Protein Identification by MALDI-TOF/TOF

Gel pieces were destained twice with 50% (v:v) aqueous acetonitrile and 25 mM ammonium bicarbonate. Gel pieces were digested at 37 °C overnight with 10 µL of trypsin solution (0.02 $\mu g.\mu L^{-1}$) in 25 mM ammonium bicarbonate, pH 8.0. Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. For each spot, the ten strongest peaks of interest, with S/N greater than 30, were selected for MS/MS

fragmentation. A peak list was generated for both the MS and MS/MS spectra by Bruker flexAnalysis software (version 3.3). Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.3), through the Bruker ProteinScape interface (version 2.1). Tandem mass spectra were searched against Viridiplantae (green plants) database with defined search parameters (15, 16).

4. Results

4.1.Oxalic Acid Concentration in S. sclerotiorum Culture Filtrate

The pH of pathogen filtrate dropped down from pH 6.0 to 3.7 after 30 days. The culture medium contained 40 mM OA. Standards and culture filtrate OA peak were detected at a retention time of 3.21–3.22 min.

4.2.Identification of Differential Protein Spots Responsive to Sunflower-S. sclerotiroum Culture Filtrate Interaction

Changes in the stem proteome of sunflower resulting from *S. sclerotiroum* culture filtrate challenge were investigated using 2-DE at 24, 48, and 72 h after treatment. Representative image was illustrated in **Figure 1** and supplementary file 1. Closer view of protein spots in susceptible and partially resistant lines at different time points compared to non-inoculated samples was shown in **Figure 2.** The features of differentially expressed proteins were presented in **Table 1**. A total of about 290 protein spots were detected on the gels in three replicates of control and treated samples of both lines. A comparison of the protein patterns between the susceptible and the partially resistant lines with respect to thier control showed that 35 spots were significantly changed in vol. % during the various time points. Out of 35 spots, 20 spots based on their expression levels were excised and subjected to MALDI TOF/TOF. Nineteen spots were successfully identified by this method (**Table 1**). The metabolic pathways changed during this interaction were summarized in **Figure 3**. The protein spots with the similar expression pattern were grouped in the same clusters.

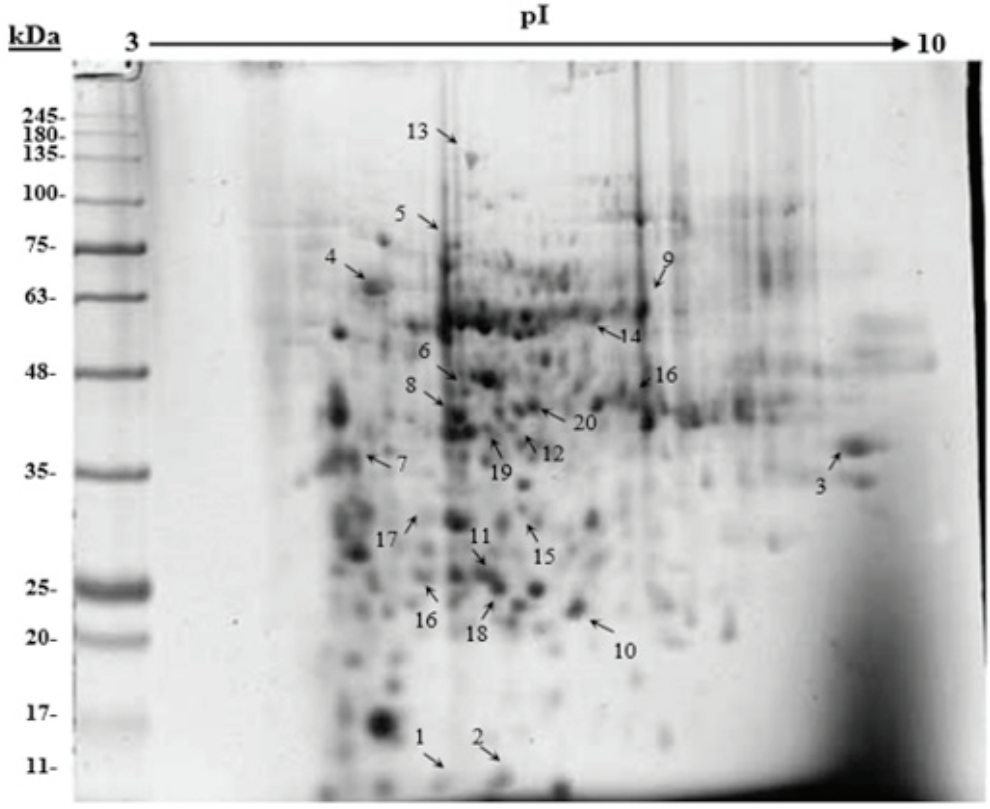

Figure 1. Representative image of partially resistant or susceptible line stem proteins separated by 2-DE. The 2-DE was performed for both lines at 24, 48 and 72 h post treatment by using 7 cm IPG strips and 12% SDS-PAGE gels. Gels were stained with Coomassie Brilliant Blue R-250. Numbers and arrows shows significantly altered spots and their descriptions have been shown in **Table 1**.

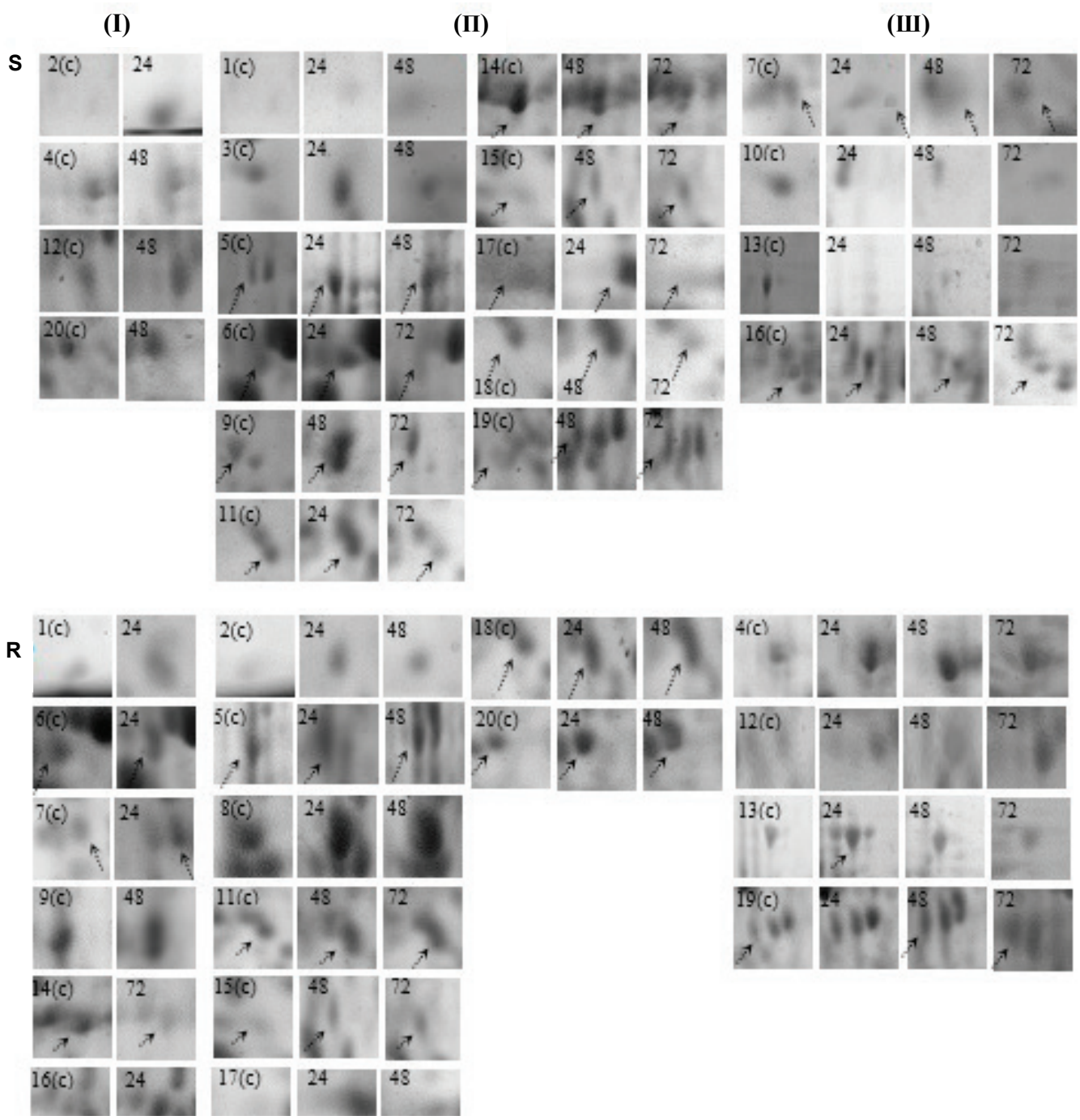

Figure 2. Closer view of protein spots in susceptible (**S**) and partially resistant (**R**) lines at different time points (24, 48 and 72 h post treatment) compared to non-inoculated samples (c). Panel (I) indicates those spots that changed at one time point. Panel (II) and (III) indicate those spots that changed at more than one time point

Table 1. Details of the proteins identified in *Helianthus annus* in response to *S. sclerotiorum* culture filtrate treatment

Protein identity	Spot ID [a]	Accession No. [b]	hpt[c]	Susceptible	Partially resistant	Mascot score [e]	MW (kDa) –pI [f]		Sequence of matched peptides
				Expression ratio (±SE) [d]	Expression ratio (±SE) [d]		Obs.	Theor.	
Cellular metabolic process									
Adenosine kinase *[Cynara cardunculus var. scolymus]*	8	976925826	24 48 72	NS NS NS	+1.6(±0.1) +1.5(±0.1) NS	331	38-5.1	33.2-5.29	K.EKPIEECVR.A K.RPENWALVEK.A R.AGCYASNVIIQR.S K.VHGWETDNVEEIAIK.I
Glutamine synthetase, beta-Grasp *[Cynara cardunculus var. scolymus]*	6	976906046	24 48 72	+1.5(±0.1) NS -1.5(±0.09)	+2.2(±0.2) NS NS	241	47-5.2	36.4-6.29	R.HTEHISAYGEGNER.R K.IIAEYIWIGGSGTDVR.S
Pyrroline-5-carboxylate reductase *[Hordeum vulgare subsp. vulgare]*	18	28628211	24 48 72	NS +1.5(±0.1) -1.8(±0.2)	+1.7(±0.1) +1.5(±0.1) NS	91	23-5.3	30.5-7.95	K.GSFRGTLINAVVA.A K.PQIVKQVLVELKPLL.S
Carbohydrate and energy metabolism									
Malate dehydrogenase *[Cicer arietinum]*	16	502155344	24 48 72	-3.6(±0.8) -4.3(±0.4)-3.8(±0.2)	+2.2(±0.1) NS NS	123	37-6.4	35.7-8.71	R.LFGVTTLDVVR.A K.ALEGADVVIIPAGVPR.K
Sucrose synthase *[Hordeum vulgare]*	20	336319004	24 48 72	NS +1.5(±0.1) NS	+1.5(±0.1) +2(±0.2) NS	65	39-5.5	34.3-5.94	K.EERAAKQRYLQMF.Y K.LGVTQGTIAHALEKT.K
Aldolase-type TIM barrel *[Cynara cardunculus]*	11	976923382	24 48 72	+1.6(±0.1) NS -1.5(±0.1)	NS +1.5(±0.1) +1.5(±0.09)	534	27-5.3	17.2-5.57	K.FFVGGNWK.C K.VAYALSQGLK.V R.IIYGGSVSGSNCK.E K.VIACVGETLEQR.E K.VASPAQAQEVHAGLR.K K.SELRPEIQVAAQNCWVK.K
Triosephosphate isomerase, cytosolic *[Tarenaya hassleriana]*	15	729290744	24 48 72	NS NS NS	NS +1.5(±0.1) +1.5(±0.1)	354	28-5.4	27.7-5.43	K.WVILGHSER.R K.VASPQQAQEVHVAVR.E
ATP synthase subunit alpha *[Helianthus annuus]*	9	575925488	24 48 72	NS +1.5(±0.1) -2.5(±0.3)	NS +1.5(±0.1) NS	402	55-6.3	47.7-6.02	R.VVSVGDGIAR.V K.AVDSLVPIGR.G R.AAELTTLLESR.I R.VVDALGVPIDGR.G K.SVHEPMQTGLK.A R.ISNFYTNFQVDEIGR.V
Photosynthesis Chlorophyll A-B binding protein *[Cynara cardunculus]*	10	976760811	24 48 72	-1.6(±0.04) -1.7(±0.1) -5.3(±0.3)	NS NS NS	89	22-6	28.2-8.67	K.VAPSGSPWYGPDR.V K.AGAQIFSEGGLDYL.G
Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit, partial *[Lasianthaea macrocephala]*	14	38146586	24 48 72	NS -3(±0.2) -3.4(±0.4)	NS NS -1.9(±0.1)	485	54-6	52.5-6.10	K.DTDILAAFR.V R.VALEACVKAR.N R.DNGLLLHIHR.A R.DLATEGNEIIR.E R.EITLGFVDLLR.D K.TFDGPPHGIQVER.D

Table 1. Table 1. Details of the proteins identified in Helianthus annus in response to S. sclerotiorum culture filtrate treatment

Protein identity	Spot ID [a]	Accession No. [b]	hpt[c]	Susceptible	Partially resistant	Mascot score [e]	MW (kDa) –pI [f]		Sequence of matched peptides
				Expression ratio (±SE) [d]	Expression ratio (±SE) [d]		Obs.	Theor.	
Redox homeostasis *Disulfide isomerase [Cynara cardunculus]*	4	976899454	24 48 72	NS -1.5(±0.1) NS	+1.9(±0.3) +2.5(±0.1) +1.5(±0.1)	155	56-4.8	55.9-4.81	K.GLAEQFEIQGFPTIK.I R.SDYDFGHTTNAELLPR.G
Stress response Gly-rich RNA binding protein *[Helianthus annuus]*	2	536461284	24 48 72	+1.7(±0.1) NS NS	+5.2(±0.4) +1.5(±0.1) NS	91	9-5.2	12.6-6.56	R.EGGYGGGGGGGYGGER.R
Voltage-dependent anion channel *[Nicotiana tabacum]*	3	161788872	24 48 72	NS +1.5(±0.09) -1.6(±0.1)	NS NS NS	181	39-8	29.5-7.74	K.ANALIQHEWRPK.S K.KGDLFLADVNTQLK.N
Peroxidase *[Helianthus annuus]*	7	56384826	24 48 72	-1.6(±0.1) -1.5(±0.1) -2.5(±0.3)	+1.5(±0.1) NS NS	325	36-4.7	34.7-8.60	R.DASVAVGGPSWTVR.L R.EMVALSGSHTLGQAR.C R.DSPDSNAAEAATDLPR.G
Protein metabolism Heat shock protein 70 *[Lactuca sativa]*	5	432140649	24 48 72	+1.5(±0.2) +1.6(±0.1) NS	+3(±0.4) +1.7(±0.1) NS	547	74-5.1	67.2-5.22	R.LDGIPPAPR.G K.DIDEVILVGGSTR.I K.QFAAEEISAQVLR.K K.AVVTVPAYFNDSQR.T R.IINEPTAASLAYGFER.K
Proteasome subunit alpha type-2-A *[Ricinus communis]*	17	255544626	24 48 72	+1.7(±0.1) NS -1.8(±0.2)	+1.6(±0.08) +1.5(±0.1) NS	99	28-5.3	25.7-5.53	R.KQAEAYHRLY.K K.GPQLYQVDPSGS.Y
Cell wall biogenesis UDP-arabinopyranose mutase 3 [Solanum lycopersicum]	12	460372644	24 48 72	NS +1.7(±0.1) NS	+1.5(±0.1) +1.6(±0.1) +1.8(±0.1)	106	40-5.4	33.8-6.25	R.HETADINTFLWGVANR.G R.DLIGPAMYFGLMGDFQPI.G
Laccase *[Solanum lycopersicum]*	13	170177495	24 48 72	-4.3(±0.5) -3.9(±0.2) -2.1(±0.4)	+3.4(±0.2) +2(±0.3) +1.5(±0.1)	101	130-5.3	63.8-8.99	R.NTIGVPVGGWAVIR.F R.FSASMNNASFQFPDKI.S
Cellular component Putative verticillium wilt disease resistance protein *[Oryza sativa subsp. japonica]*	19	55296769	24 48 72	NS +1.5(±0.1) +1.7(±0.09)	+1.6(±0.1) +2(±0.09) +1.7(±0.1)	65	40-5.3	39.4-5.65	R.VLILRSNQFYGSVG.L R.SFEGNAGLCGRPLSKQ.C
other Single peptide match to hypothetical protein JCGZ_14654 *[Jatropha curcas]*	1	643717257	24 48 72	+1.8(±0.9) +1.9(±0.1) NS	+1.6(±0.1) NS NS	57	9-4.9	50.5-8.16	K.ALMEEVK.K

NS: non-significant

Up regulated protein
Down regulated protein

Table 1. [a] Spot number as shown on the 2-D gel image (**Fig. 1**)
[b] Accession number obtained from NCBI database was assigned to each protein.
[c] hpt is hours post treatment.
[d] Expression ratios or fold changes for each spot was equal to the relative expression (average of spot vol.% values of treatment with respect to their control) at each time point. The responses are considered as *S. sclerotiorum* responsive ones when the value of fold changes is ≥ 1.5. SE represents the standard error of expression ratio at each time point.
[e] Score>50 are significant ($p<0.05$).
[f] Theoretical molecular mass and isoelectric point were obtained from Mascot search results, protein view and experimental molecular mass and isoelectric point were obtained from 2-DE gel image analyzed by Imagemaster software.

In susceptible line, spots 7, 10, 13 and 16 were down-regulated (1.6, 1.6, 4.3 and 3.6-times) at all time points. Spots 1, 5 and 19 were up-regulated at two time points after culture filtrate treatment. The abundance of spots 3, 9 and 18 was not reproducibly changed at 24 h, but their abundance was increased at 48 h (1.5, 1.5 and 1.5-times) and then decreased at 72 h (1.6, 2.5 and 1.8-times) after treatment. Proteins 6, 11 and 17 were found to be up-regulated (1.5, 1.6 and 1.7-times) at an earlier time point (24 h), but then down-regulated (1.5, 1.6 and 1.8-times) at a later time point (72 h). Proteins 2, 4, 12 and 20 were observed to be either up- or down-regulated only at one time point. Spot 14 was down-regulated (3 and 3.4-times) at two time points (48 and 72 h) after culture filtrate treatment. The abundance of spot 8 and 15 was not reproducibly changed at all sampling times. In the partially resistant line, spots 4, 12, 13 and 19 were up-regulated at all time points. Spots 2, 5, 8, 17, 18 and 20 were up-regulated at 24 and 48 h and spots 11 and 15 were also up-regulated at two time points 48 and 72 h after treatment (**Table 1**). Protein 14 was found to be down-regulated (1.9-times) at the later time point (72 h). Proteins 1, 6, 7, 9 and 16 were observed to be up-regulated (1.6, 2.2, 1.5, 1.5 and 2.2-times) only at one time point. The abundance of spot 3 and 10 were not reproducibly changed at all sampling times.

4.3. Functional Classification of Identified Proteins and Biological Significance

Based on the available literature and protein database InterPro (http://www.ebi.ac.uk/interpro), the identified proteins were classified into several groups (**Fig. 4**). It was obvious that a large portion (25%) of the identified proteins involved in carbohydrate and energy metabolism. The second largest class of proteins (15%) was identified as those with a role in stress response and cellular metabolic process. Other categories (10%) consist of proteins having roles in photosynthesis, protein metabolism and cell wall biogenesis. The remainder played a role in redox homeostasis (5%). The identified proteins might have major roles in mediating sunflower responses to *S. sclerotiorum* attack.

5. Discussion

There are many types of research that indicate the ability of proteomics studies in unraveling novel information about cellular processes that occur in the plant under abiotic and biotic stresses (15-18, 27, 28, 29). In the present study, proteomics-based analysis involving 2-DE analysis was conducted to get a better understanding of the defense mechanisms in stem tissues of partially resistant and susceptible lines of sunflower against culture filtrate of *S. sclerotiorum*. The proteins that were identified in the current investigation involved in a wide variety of processes including photosynthesis, carbohydrate metabolism, redox homeostasis, plant cell wall biogenesis, stress response, metabolic process and protein processing.

5.1. Photosynthesis-Related Proteins

In this study, two protein spots were identified as photosynthesis-related including the Rubisco large subunit and chlorophyll A-B binding protein. Chlorophyll A-B binding protein was downregulated at all sampling times in susceptible line and Rubisco was observed to be downregulated in susceptible line at 48 and 72h and in partially resistant line at 72 h post treatment. Degradation and downregulation of photosynthetic proteins in response to stresses such as OA have also been reported in previous studies (16, 30). Yang *et al.* (30) presented two possible mechanisms by which *S. sclerotiorum* infection damage the photosynthetic apparatus of tobacco leaves. They presumed that $C_2O_4^{2-}$, anion group of oxalic acid, negatively affects D1 synthesis, a protein required for repairing PSII photoinhibition. Yang *et al.* (30) also suggested that $C_2O_4^{2-}$ anion may chelate Mg^{2+} in chloroplast that subsequently decrease

Rubisco activity in plant cells. Decrease in the levels of these proteins may cause decrease in photosynthetic efficiency that may contribute to negative effect in overall ability of these plants to defeat the pathogen. Obviously, in the susceptible lines the photosynthetic performance would be less, particularly in necrotic tissues. In accordance with previous studies (16, 30), OA might decrease the expression of photosynthesis-related proteins particularly in susceptible line, which has a negative impact on host photosynthetic capacity.

5.2. Carbohydrate and Energy Metabolism

Aldolase-type TIM barrel has a role in carbohydrate metabolic process and pentose phosphate pathway that provide energy for defense responses. Vanholme *et al.*, (31) also demonstrated the involvement of aldolase in lignification and phenolic metabolism. In the current study, aldolase-type TIM barrel was up-regulated at 48 h and 72 h post treatment in partially resistant plant and in the susceptible plant it was up-regulated at 24h and then down-regulated at 72h. Moreover, malate dehydrogenase (MDH) and triosephosphate isomerase were differentially expressed in the two lines. The abundance of these enzymes was increased only in the partially resistant line in response to pathogen filtrate challenge. The other proteins that involved in carbohydrate and energy metabolism were ATP synthase and sucrose synthase. The expression of ATP synthase was up-regulated in both susceptible and partially resistant lines at 48 h post treatment, and then it was sharply down-regulated at 72 h post treatment in susceptible line. The abundance of sucrose synthase was increased gradually in the partially resistant lines at 24 and 48h post treatment, while in the susceptible line it was only up-regulated at 48h.

These data reveal the role of primary metabolism during sunflower- *S. sclerotioum* interaction. The two enzymes, malate dehydrogenase and ATP synthase subunit alpha, involved in carbohydrate and energy metabolism are essential to retain the metabolic state of the diseased tissues probably by regulating the TCA cycle and electron transport chain (32). Coordinated regulation of genes and metabolites involved in the TCA cycle and glycolysis pathway has been observed in previous studies (32). More increase in the expression of the enzyme with a role in glycolysis and pentose phosphate pathways such as aldolase, triosephosphate isomerase and sucrose synthase and differentially expression of the enzyme with a role in TCA cycle enzymes such as malate dehydrogenase in partially resistant line could participate in providing energy for cytoskeleton reorganization during defense responses and subsequently increase tolerance and total adaption of partially resistant line to *Sclerotinia* infection. Moreover, higher expression of aldolase might impact in the degree of lignification of cell wall in partially resistant line.

5.3. Metabolic Process

The Adenosine kinase (ADK) was only up-regulated in partially resistant line. Liu *et al.*, (33) showed that ADK is one component of basal defense responses in wheat against stripe rust disease. It was demonstrated that ADK plays key roles in mediating sugar and energy metabolism in cells under stresses. Its activity also has a positive effect on level of methyl-esterified pectin (33). Cell wall methyl-esterified pectin show more resistant to degradation by fungal pectolytic enzymes (33). Therefore, it was speculated that ADK might impact on methyl transfer reactions and energy metabolism in favor of plant defense mechanisms in partially resistant line.

Pyrroline-5-carboxylate reductase catalyzes the terminal step in the biosynthesis of proline (34). Based on the present study, the abundance of this protein was increased at 24 and 48 h in partially resistant line and this increase was observed in the susceptible line at 48 h but was sharply decreased at 72 h after treatment. In accordance with this results, we observed that the proline content in the stem of partially resistant line was higher than that in the susceptible line (26). Proline contributes to the defense responses by several mechanisms including increasing the activity of antioxidant enzymes such as CAT, POX, SOD, glutathione-S-transferase, involvement in cell redox homeostasis and direct ROS scavenging activity (35, 36, 37). Therefore, it seems that proline is essential for plant recovery from stress.

Glutamine synthetase (GS) in conjunction with glutamate synthase contribute to the incorporation of ammonia into amino acids such as proline (38). The abundance of this protein was increased at 24 h after treatment in the partially resistant and susceptible line but then its expression was sharply decreased at 72 h post treatment in the susceptible line. GS has been shown that plays a role in the synthesis of the main phytoalexin of Arabidopsis, camalexin (Bohman *et al*., 2003). Changes in the expression of this protein in response to pathogen filtrate might be related to defense responses in sunflower. However, the precise role of this protein in the tolerance of sunflower to *S. sclerotirium* needs to be further studied.

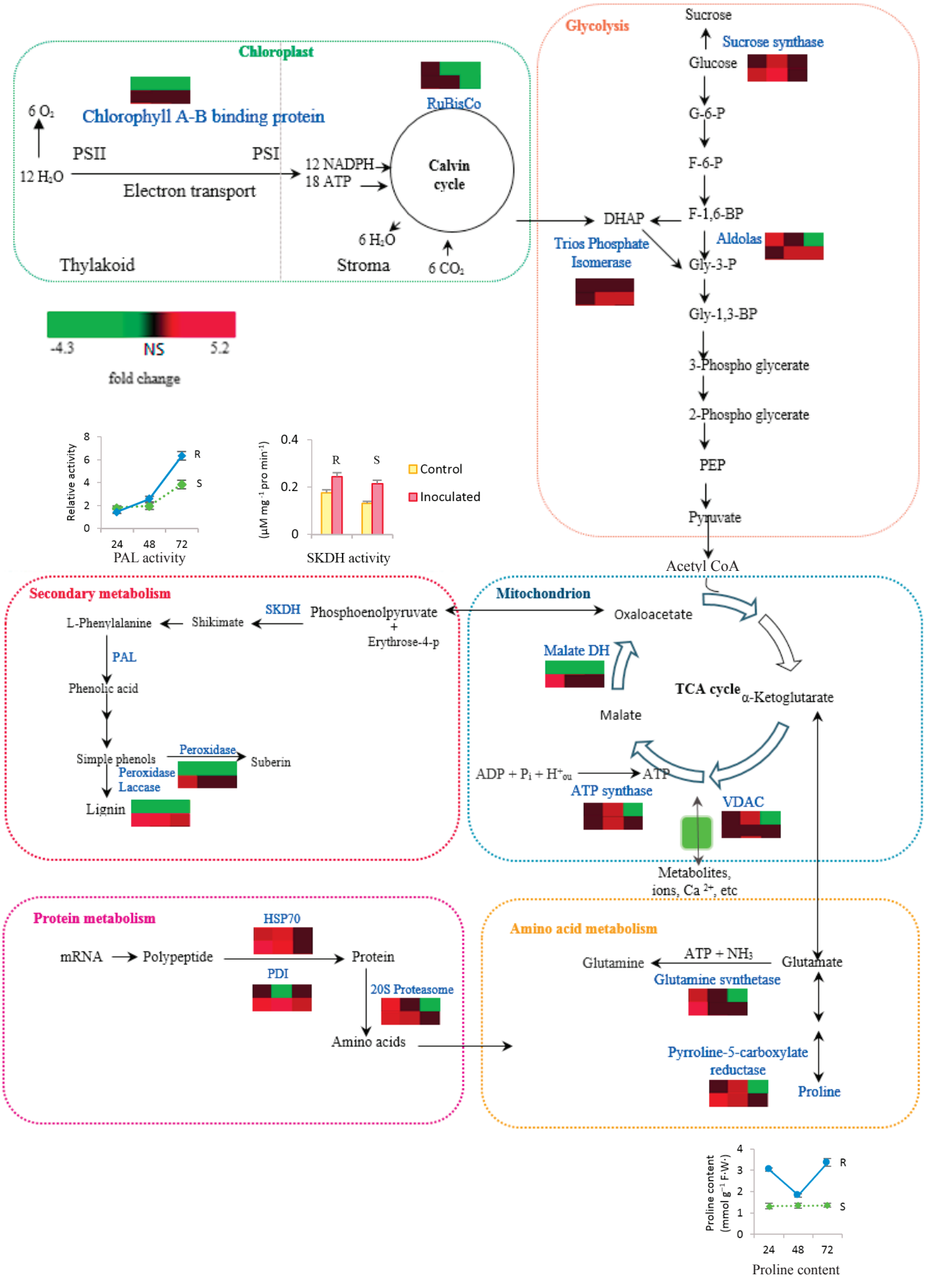
Chloroplast
Chlorophyll A-B binding protein
RuBisCo
6 O2
PSII
PSI
12 H2O
Electron transport
12 NADPH
18 ATP
Calvin cycle
6 H2O
6 CO2
Thylakoid
Stroma
-4.3
NS
5.2
fold change
Glycolysis
Sucrose
Sucrose synthase
Glucose
G-6-P
F-6-P
F-1,6-BP
Aldolas
DHAP
Trios Phosphate Isomerase
Gly-3-P
Gly-1,3-BP
3-Phospho glycerate
2-Phospho glycerate
PEP
Pyruvate
Acetyl CoA
Relative activity
PAL activity
(µM mg-1 pro min-1)
SKDH activity
Control
Inoculated
Secondary metabolism
SKDH
Phosphoenolpyruvate + Erythrose-4-p
L-Phenylalanine
Shikimate
PAL
Phenolic acid
Simple phenols
Peroxidase
Suberin
Peroxidase
Laccase
Lignin
Mitochondrion
Oxaloacetate
Malate DH
Malate
TCA cycle
α-Ketoglutarate
ADP + Pi + H+ou
ATP
ATP synthase
VDAC
Metabolites, ions, Ca 2+, etc
Protein metabolism
HSP70
mRNA
Polypeptide
Protein
PDI
20S Proteasome
Amino acids
Amino acid metabolism
ATP + NH3
Glutamine
Glutamate
Glutamine synthetase
Pyrroline-5-carboxylate reductase
Proline
Proline content (mmol g-1 F.W.)
Proline content

Figure 3. Main metabolic pathways changed in sunflower stem in response to virulence factors of *S. sclerotiorum.* The color graphs indicate fold change of identified proteins at various time points. Columns from left to right represent results obtained from various sampling times (24, 48 and 72 h after treatment). The first row represents results for the susceptible line and the second row represents results for the partially resistant line. Color scales of fold change values are shown, in which red and green colors indicate the higher and lower expression levels, respectively. Column chart shows SKDH activity in partially resistant (R) and susceptible (S) lines after treatment by pathogen culture filtrate (25). Line graph shows relative activity of PAL and column chart links to proline content in S and R lines at various time points (26). R, partially resistant line AC4122; S, susceptible line HA89; DHAP, dihydroxy acetone phosphate; HSP70, heat shock 70 kDa protein; Malate DH, malate dehydrogenase; PAL, phenylalanine ammonia lyase; PDI, protein disulfide-isomerase; SKDH, shikimate dehydrogenase; VDAC; voltage-dependent anion channel; NS, non-significant.

5.4. Plant Cell Wall Biogenesis

In this study, comparative analyses between partially resistant and susceptible lines of sunflower revealed substantial differences in the abundance level of laccase, the enzyme that has been associated with lignin polymerization (39). Up-regulation of this protein in partially resistant line and its down-regulation in susceptible line at all sampling times might provide evidence that cell wall reinforcement plays a role in the resistance of sunflower to *S. sclerotiorum*.

Protein No. 12 was identified as arabinose mutase III. This protein has a role in the synthesis of xyloglucan. It was suggested that this protein participates in plant defense responses (28, 40-41). Li *et al.* (40) also reported that the expression of UDP-forming was only increased in highly resistant banana cultivar following *Fusarium oxysporum* f. sp. *cubense* inoculation. In the present study, this protein was up-regulated sharply at all sampling times in partially resistant line, while this increase was only observed at 48 h post treatment in susceptible line. Strengthen of plant cell wall might help prevent oxalic acid penetration in plant cell and improve resistance mechanism of sunflower against disease.

In the present study, the expression of enzymes involved in strengthening plant cell wall including laccase, arabinose mutase III, aldolase, ADK and peroxidase were higher in partially resistant line that those in the susceptible one. Induction of cell wall metabolism and formation of the complex cell wall may contribute to sunflower defense strategies and reducing symptom development by strengthening plant cell wall against pathogen penetration. In this study, this strategy was more obvious in partially resistant line than susceptible line.

5.5. Redox Homeostasis

The protein disulfide isomerase (PDI) is well known for its role as chaperones in formation, reduction and rearrangement of disulfide bonds in the endoplasmic reticulum (42-44). PDI catalyzes the cleavage of disulfide bonds under oxidative stress and regulates the intracellular redox balance. Antioxidant enzymes are turned into an active form by reduction of the disulfide bonds during antioxidant defense (45, 46). Ondzighi *et al*., (47) indicated that loss of expression of *PDI5* caused premature induction of PCD during embryogenesis. Ray *et al.* (48) suggested that PDI may play critical role in host plants to suppress the very highly harmful and energy-consuming processes during programmed cell death (48). In the current study, the abundance of PDI was found to be increased only in partially resistant line. Therefore, antioxidant properties of PDI may help limit potential cell injury resulting from ROS generated by *Sclerotinia*-secreted OA.

5.6. Stress Response

Gly-rich RNA binding proteins (GRPs) participate in gene expression regulation at the post-transcriptional level in response to various stresses and play a crucial role in plant innate immunity (49). Wang *et al.* (50) reported that GRPs could regulate plant physiological changes and enhance the activities of superoxide dismutase (SOD) and catalase (CAT) upon salinity stress. In our study, GRP abundance was more induced by pathogen filtrate treatment in partially resistant line. There's a possibility that the presence of OA in culture filtrate caused oxidative damage in plant tissues, which in turn the expression of GRP was induced in sunflower lines and help to decrease oxidative stress in plant tissues.

In our previous study, we observed that the activity of antioxidant enzymes namely SOD, POX, CAT and DPPH radical scavenging were higher in sunflower partially resistant line or activated earlier compared to the susceptible one (26). In the present study, some proteins that were expressed differentially in the two sunflower lines including pyrroline-5-carboxylate reductase, glycine rich protein (GRP) and disulfide isomerase (PDI) may help to clarify this difference in

the efficiency of the antioxidant enzyme system. These proteins might impact positively on the activities of antioxidant enzymes in partially resistant line that helped to remove the ROS induced by *Sclerotinia*–secreted OA. Subsequently, it might delay PCD and slow the growth and development of this necrotrophic pathogen in sunflower tissues.

Anion effluxes are mediated by anion channel activity (51). The activation of an anion channel was shown to be essential components of OA-induced PCD in *Arabidopsis thaliana* plant cells (51). In our study, the abundance of voltage-dependent anion channel was increased at 48 h and then decreased at 72h in susceptible line, while its expression was not changed reproducibly in partially resistant line at all sampling times. We presumed that increased levels of this protein in susceptible line might be involved in the induction of PCD in the susceptible line. However, the exact role of this protein in enhancing susceptibility of sunflower to *S. sclerotirium* needs to be further studied. Fluctuation in the abundance of this protein in susceptible line could point out to alterations of mitochondrial function in response to pathogen filtrate treatment. Whereas, it seems that in partially resistant line formation of the mitochondrial permeability transition pore was not affected by pathogen filtrate treatment.

The other protein that was classified in stress response category was peroxidase. Both acidic and basic peroxidase isozymes are involved in lignification and plant defense responses against pathogens (52). It has been reported that the acidic peroxidase contributed to the suberization in potato tubers during wound healing (52). In our study, a peroxidase isoform showed a pI of 4.7 on the 2DE gel. The expression of this protein was increased at 24 h after treatment in partially resistant line, while in susceptible line was decreased at all sampling times. Besides the involvement of peroxidases in antioxidant defense system and lignification of the cell wall, it is also possible that suberin layer has a role in tolerance of sunflower against Sclerotinia penetration.

5.7. Protein Metabolism

Heat shock proteins (HSP70) was identified as a molecular chaperone, which was induced by pathogen filtrate treatment at 24 and 48 h post treatment in both lines. However, it was more pronounced in partially resistant line. HSP70 plays an important role in stabilizing new proteins to establish proper folding or by assisting to refold proteins that were misfolded by the stresses (53). Therefore, the synthesis of this protein could be an adaptive response to the pathogen filtrate treatment in both lines.

The level of 20S proteasome alpha subunit B1 (PAB1) was significantly changed after treatment with culture filtrate. In the susceptible line, its expression was increased at 24 h and then decreased at 72 h post treatment. While, in partially resistant line it was increased at 24 and 48 h post treatment. (54). It was demonstrated that increased 20S proteasome biogenesis raises the capacity of cell to breakdown damaged and oxidized proteins and thus enhances tolerance to oxidative stress (55). Gene silencing

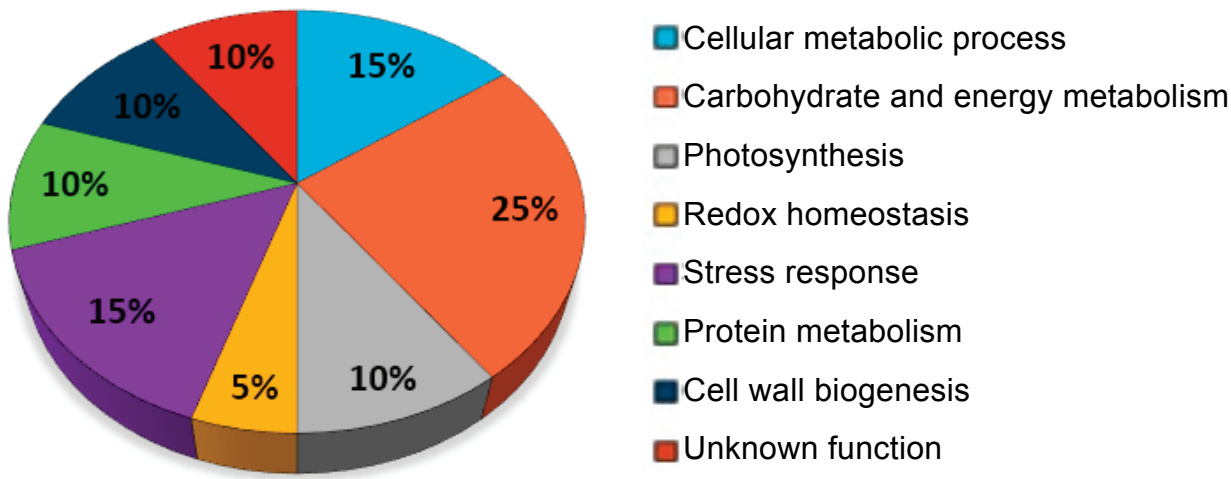

Figure 4. The functional classification of the identified proteins

of PAB1 reduced its proteasome activity, caused DNA fragmentation, enhanced ion leakage and ROS production (55). The up-regulation of this protein in partially resistant line at two sampling time might improve tolerance of sunflower to oxidative stress caused by *Sclerotinia*-secreted OA, while its down-regulation in susceptible line could lead to degradation of the metabolic pathway under pathogen filtrate treatment.

In the present study, down-regulation of PAB1 and up-regulation of voltage-dependent anion channel expression in susceptible line might lead to ion leakage, production of ROS and thus increased the susceptibility of plant to this pathogen.

5.8. Unknown Function

Two proteins with unknown function, including putative verticillium wilt disease resistance protein and a hypothetical protein were found to be up-regulated in susceptible and partially resistant lines. Putative verticillium wilt disease resistance protein is a protein with leucine-rich repeat (LRR) and is an integral component of the membrane. This protein was up-regulated earlier in partially resistant line at 24 h post treatment and then its expression was increased in both lines at 48 and 72 h after treatment. Up- regulation of disease resistance protein (TIR-NBS-LRR class) and gene encodes for a leucine-rich repeat (LRR) family protein have been reported in both resistant and susceptible genotypes of *B. napus* under *S. sclerotiorum* inoculation (56). Characterizing these proteins and understanding their possible roles in defense mechanisms may help to answer several questions regarding both compatible and incompatible sunflower-*S. sclerotiorium* interaction.

6. Conclusion

In conclusions, our observations imply that degree of cell wall pectin methylation, suberification, lignification and polysaccharide production may play essential roles in the resistance mechanisms of sunflower against the pathogen. The functional characterization of these proteins could lead to the improvement of sunflower lines with higher levels of resistance to *S. sclerotiorum*. In addition, higher expression of pyrroline-5-carboxylate reductase, PDI, GRP and HSPs might cause more efficient antioxidant system in partially resistant line. Down-regulation of some protein such as PAB1 might increase the susceptibility of plant to this pathogen. Our findings showed that some proteins involved in carbohydrate metabolism (malate dehydrogenase, triosephosphate isomerase), plant cell wall biogenesis (laccase), metabolic process (adenosine kinase), stress response (peroxidase) and regulating cell redox homeostasis (disulfide isomerase) were only up-regulated in partially resistant line. These proteins may convey resistance to *S. sclerotiorum* in sunflower. Over-expression of the enzymes that were specifically up-regulated in the resistant genotype would be an efficient strategy for improving plant resistance against this pathogen. In addition, detailed characterization of proteins that were identified in this study may reveal potential metabolic pathways for engineering of resistance to this devastating necrotrophic pathogen.

Acknowledgements

The authors thank Iran National Science Foundation (INSF) (grant number 97011209) and National Institute of Genetic Engineering and Biotechnology, NIGEB (Project No. 785) for funding this research program.

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