*Trends in Horticulture* 2025, 8(2), 11123.
https://doi.org/10.24294/th11123

Article

# Proteomic insights into the promotive effect of *Pseudomonas fluorescens* FY32 on canola (*Brassica napus* L.) under salt stress

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**CITATION**

Banaei-asl F, Bandehagh A, Uliaei E D, et al. (2025). Proteomic insights into the promotive effect of *Pseudomonas fluorescens* FY32 on canola (*Brassica napus* L.) under salt stress. Trends in Horticulture. 8(2): 11123. https://doi.org/10.24294/th11123

**ARTICLE INFO**

Received: 29 December 2024
Accepted: 14 July 2025
Available online: 23 December 2025



**Abstract:** A comprehensive proteomic analysis was carried out to evaluate leaf proteome changes of Brassica napus cultivars as an important oilseed crop inoculated with the bacterium Pseudomonas fluorescens FY32 under salt stress. Based on the physiochemical characteristics of canola, Hyola308 was a tolerant and Sarigol was a salt sensitive cultivar. Gel-based proteomics indicated that proteins related to energy/metabolism, cell/membrane maintenance, signalins, stress, and development respond to salt stress and bacterial inoculation in both cultivars. Under salt stress, Hyola308 launches mechanisms similar to Sarigol, but the tolerance was related to consuming less energy consumption than Sarigol for launching the proper pathway/mechanism. Inoculation with plant growth promoting bacteria promotes relative growth rate and net assimilation rate; causes increase in soluble sugar content (12–32% varing to cultivars and salt treatments), as an osmo-protectant, in leaves of Sarigol and Hyola308 in control and salt stress conditions. The groups of proteins that are affected due to inoculation (18 and14 functional groups in Hyola308 and Sarigol, respectively) are varying to stress-influenced groups (10 and 6 functional groups in Hyola308 and Sarigol, respectively) that might be because of regulating tolerance mechanism of plant and/or plant-growth promoting bacteria inoculation. Furthermore, it is recognized that P. fluorescens FY32 has a dual effect on the cultivars including a pathogenic effect and a growth promoting effect on both cultivars under salt stress.

**Keywords:** proteomics; canola; plant growth promoting bacteria; salt stress

## 1. Introduction

Canola (*Brassica napus*), previously known as rapeseed, is one of the most commonly grown oil seeds [1]. Additionally, canola is attracting attention as biofuel source and as animal feed [2]. The vast majority of agricultural land undergoes abiotic stress that can significantly reduce agricultural yields [3]. The ability of plants in water absorbtion reduces at a high salt concentration in soil, referred to as the water-deficit effect of salinity. Osmotic stress cases the same metabolic changes in plant as those caused by water stress, with little difference between genotypes [4]. ion toxicity occurres due to the accumulation of phytotoxic ions, especially $Na^+$ and $Cl^-$ which tolerant genotypes can prevent salt from building up to toxic levels in leaves [5,6], which leads to a wide variety of changes such as morphological, biochemical, and

physiological at cellular, tissue, organ and whole plant levels [7]. Although canola is considered to be a moderately salt-tolerant plant, comparative salt-tolerant studies have noted that some varieties of this plant exhibit significant salt tolerance during early stage of growth [8].

Rhizobacteria as root-colonizing bacteria that form endophytic relationships with many plants, are capable of increasing plant growth and yield and give them the ability to resist harsh environmental conditions better than non-inoculated plants [9]. Plant Growth-Promoting Rhizobacteria (PGPR) have several useful effects on plant physiological responses to the environment such as fixation of nutrients, decrease of the toxicity of heavy metals, control of soil-borne pathogens, and alteration of plant-hormonal balance [9]. Plant-colonizing microbes have received increasing attention in recent years since they can boost plant growth and yield and can strengthen plant responses to abiotic stress [10]. Cheng et al. [11] studied the effects of two strains of *Pseudomonas putida* UW4 (wild type UW4 and AcdS minus mutant) on the proteomic profile of *B. napus* under salt stress. They were reported that the presence of both salt stress and bacterial inoculation affects the abundance of 76 different proteins. Many of the identified proteins were involved in photosynthesis, anti-oxidative processes, transportation across membranes, and pathogenesis-related responses. *Pseudomonas fluorescens* (*P. fluorescens*) is a PGPR and a member of a group of common, nonpathogenic saprophytes colonizing soil, water and plant environments [12]. In the previous study, as a part of an effort to obtain an ACC deaminase containing bacteria from a collection of soil samples, *P. fluorescens* FY32 exhibited only a single PGPR attribute—ACC deaminase activity [13].

The effects of salinity stress and inoculation of bacteria on the root of these cultivars have also been studied using gel-based proteomics, proteins related to amino acid metabolism and tricarboxylic acid cycle were affected in both sensitive and Hyola308 [14]. Meanwhile, shotgun proteomic analysis on leaves of sensitive and tolerant cultivars of canola indicated that the level of copper/zinc superoxide dismutase 1 protein was significantly increased in Hyola308 inoculated under severe salt stress (300 mM NaCl) and decreased under 150 mM NaCl [15].

In this experiment, salt-tolerant and -sensitive canola cultivars were subjected to saline conditions to evaluate the effect of salt stress on the physiochemical traits of canola. The traits soluble sugars and chloride ion were evaluated both in non- and inoculated cultivars under salt stress. Additionally, a gel-based proteomic analysis was performed to study the molecular mechanisms of PGPR's beneficial effects on inoculated canola cultivars under salt stress.

## 2. Materials and methods

### 2.1. Plant materials

The seeds of canola (*B. napus* L. cultivars. Hyola308 and Sarigol) were provided from Seed and Plant Improvement Institute, Karaj, Iran. After sterilization of identical homogenous seeds [16] and germination under aseptic conditions, 2-week-old seedlings were transplanted in a hydroponic system and were fed using the sterilized Hoagland's solution [17]. The plants were cultivated with 3 replications in the plastic pipes full of sterile sands with the total volume of 40 L. Each replication was used for

10 plants. The Hoagland's solution was flushed on the sands by the air pump every 2 h. The condition of greenhouse was controlled to have the temperature in a range of 25±2 °C during the day and night and relative humidity of about 50% during the day (14 h) and 60% at night (10 h). Light (with approximately Photon flux density of 1800 µmol. $M^{-2}$. $s^{-1}$) and pH of nutrient solution (6.5±0.5 using hydrochloric) were regulated (**Figure S1**).

## 2.2. Bacteria preparation

The strain *P. fluorescens* FY32 [13] was aerobically cultured in 250 mL of LB medium [18] in a shaker incubator at 30 °C for 18 h. Centrifugation at 8000× g for 10 min at 4 °C was applied to harvest the bacteria. The supernatant was removed, and the cells were re-suspended in 25 mL of sterile 0.03 M $MgSO_4$, and then placed on ice [13]. McFarland's procedure [19] was utilized to determine the population of cultured bacteria. A sample (0.5 mL) was removed from the cell suspension and serially diluted in sterile 0.03 M $MgSO_4$. The absorbance of the sample was measured at 600 nm. This measurement was used to adjust the turbidity of bacterial suspensions so that the number of bacteria reached around $10^{10}$ cfu $mL^{-1}$.

## 2.3. Applying stress conditions and bacteria inoculation

Before inserting seedlings into the hydroponic system, 10 mL of bacterial suspension in 30 mM $MgSO_4$ was inoculated to each tank containing 10 L of nutrient solution [20]. After ensuring of the establishment of 14-day-old seedlings in the system, salinity stress in 300 mM NaCl was applied in a split-split plot based on a completely randomized experimental design. Reestablishment of Nutrient solution, bacteria suspension, and NaCl treatment, which was put in effect every 14 days to maintain the treatments at the same level, were applied.

## 2.4. Relative growth rate and net assimilation rate

The samples for measuring relative growth rate (RGR) and net assimilation rate (NAR) were measured simultaneous with stress induction (i.e. $t_1$ at which the plants were 21 days old) and when the plants were 51 days old ($t_2$). Three plants were selected from each replication and dried in a hot air oven at 80 °C for 72 h. The dry weight of the samples was recorded and the average of each treatment was used to calculate RGR and NAR. These characteristics were calculated as follows:

$$\text{RGR} = \frac{\ln W2 - lnW1}{t2 - t1} \ (\text{g.g}^{-1}.\text{d}^{-1})$$

Where $W_1$ and $W_2$ are plant dry weights at $t_1$ and $t_2$. $t_1$ and $t_2$ are the times for the harvest when the plants were 21 and 51 days old, respectively.

$$\text{NAR} = \left(\frac{W2 - W1}{t2 - t1}\right)\left(\frac{lnA2 - lnA1}{A2 - A1}\right) \ (\text{g m}^{-2}\ \text{d}^{-1})$$

Where $W_1$ and $W_2$ are plant dry weights at $t_1$ and $t_2$. $A_1$ and $A_2$ represent leaf area at $t_1$ and $t_2$, which was measured using leaf area meter Model LI-3100C (LI-COR Biosciences, Lincoln, Nebraska, USA) at $t_1$ and $t_2$, respectively.

## 2.5. Measurement of soluble sugar and chloride ion contents

To evaluate the effects of NaCl and *P. fluorescens* FY32 presence in canola rhizosphere, extraction of soluble carbohydrates of fresh leaf tissues was done using 70% ethanol. Total soluble sugars were determined using the phenol/sulfuric acid method [21], then the absorbance at 485 nm was determined. Using glucose standard curve, total soluble carbohydrate contents were estimated. To chloride ion content determination [22], Oven dried tissue (50 mg) was grounded and 10 mL of distilled water was added on it. Then put into boiling water for 15 min. After that, the mixture was leached till 5 mL of clear solution was gained. 2 mL of iron nitrate solution (2.02 g of iron (III) nitrate in 98 mL of distilled water and 2 mL of 0.2 N nitric acid) and 2 mL of saturated solution of Mercury thiocyanate (0.75 g of mercury (II) thiocyanate in 100 mL of distilled water) was added. After 15 min, the absorbance at 460 nm was determined.

## 2.6. Protein extraction

A portion of Leaves (500 mg) from 51-day-old plants was ground in liquid nitrogen using a mortar and pestle. Acetone with 10% trichloroacetic acid and 0.07% 2-mercaptoethanol were added to the resulting powder. It was sonicated for 10 min, incubated for 1 h at −20 °C, and centrifuged at 9000× g for 20 min at 4 °C. The supernatant was casted-off and the pellet moved into a solution of 0.07% 2-mercaptoethanol in acetone for 1 h. Another centrifugation at 20,000× g for 5 min at 4 °C for the suspension was performed and washed twice using 0.07% 2-mercaptoethanol in acetone. The final pellet was dried using a Speed-Vac concentrator (Savant Instrument, Hicksville, NY, USA) and re-suspended in lysis buffer containing 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine. The protein concentration was measured by the Bradford method [23] with bovine serum albumin as the standard.

## 2.7. Two-dimensional polyacrylamide gel electrophoresis and gel-image analysis

Extracted proteins (400 µg) were separated with two-dimensional polyacrylamide gel electrophoresis (2-DE) (**Table S1**). The images of 2-DE were acquired using a GS-800 calibrated densitometer scanner (Bio-Rad). The position of individual proteins on each gel was assessed by PDQuest software (version 8.0; Bio-Rad). The pI and molecular mass of each protein were determined using a 2-DE marker (Bio-Rad). The amount of a protein spot was expressed as the volume of that spot, which was defined as the sum of the intensities of all the pixels, which make up that spot. To compare spots between gels, spot volumes were normalized as a percentage of the total volume in all spots. Spots with significant changes in volume were identified using the *F*-test through analysis of variance ($p < 0.05$) (**Figure S2**).

## 2.8. Protein preparation and data acquisition by nano-liquid chromatography-mass spectrometry

For gel-based proteomic analysis, spots were cut-off from the CBB-stained gels and washed with water. Proteins in the excised gel pieces were prepared for nano-

liquid chromatography mass spectrometry (LC MS/MS) with an Ultimate 3000 nano-LC system and a nanospray LTQ XL Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA) (**Table S1**). After LC MS/MS analysis, the obtained MS and MS/MS spectra were used to identify differentially abundant canola proteins using the MASCOT search engine (version 2.4.1; Matrix Science, London, UK) and Protein Discoverer (version 1.4.0.288, Thermo Fisher Scientific) against Arabidopsis peptide database (TAIR10, http://www.arabidopsis.org/; 35,386 entries) (**Table S1**).

### 2.9. Analysis of protein function

Protein functions were predicted and categorized using MapMan Bin codes [24,25] and Mercator tool [26] for plant protein categorization.

### 2.10. Statistical analysis

The statistical significance of the results was evaluated by the Student's *t*-test when only 2 groups were compared. All the measurements and analyses were given with 3 biological and technical replications. The statistical significance of the multiple groups was evaluated with One-way ANOVA test. A $p$-value <0.05 was considered statistically significant. The Analysis related to assumptions of ANOVA have done before that. All calculations were performed using software SPSS and MS-TATC. Cut-off parameters for the identified proteins were $p$-value <0.05 with 2.0 in fold change.

## 3. Results and discussion

### 3.1. Physiochemical characterization

RGR and NAR**:** Based on the analysis of variance of RGR and NAR, significant differences among cultivars, bacterial inoculation, and salt-stress treatments, were analyzed (**Table 1**). Hyola308 had more of the tolerant cultivar RGR than the sensitive cultivar Sarigol, whereas Sarigol decreased in both traits under salinity stress without bacterial inoculation (**Figure 1A**). Hyola308 had higher RGR levels than Sarigol, and bacterial inoculation enhanced RGR in both salt stress and control conditions. Therefore, inoculated plants are better than non-inoculated ones in terms of RGR amount. Several studies reported similar trends in growth parameters under salt stress in other plant species such as naked oats, rice, kenaf, barley, and wild species, as well as canola under drought stress [27–32]. The change in NAR values is almost similar to that of RGR. Comparing the mean values of NAR, Hyola308 presented higher NAR values than Sarigol. According to the above parameters, inoculated plants performed better than non-inoculated plants and exposure to salt stress resulted in a significant decline in the NAR values of both varieties (**Figure 1B**). The net gain in biomass results from $CO_2$ assimilation by leaves minus respiratory loss by the entire plant (Wendering and Nikoloski 2023). Therefore, leaf area can be viewed as a powerful variable considered by calculating NAR as another trait for evaluating plant growth under salt stress. NAR represents the net photosynthetic efficiency of a plant's ability to capture light, assimilate $CO_2$, and store photosynthetic products. Variation in NAR can be caused by differences in canopy structure/light absorption, leaf photosynthetic

activity, respiration, photosynthate transport, and sink storage capacity, as well as the chemical nature of stored products.

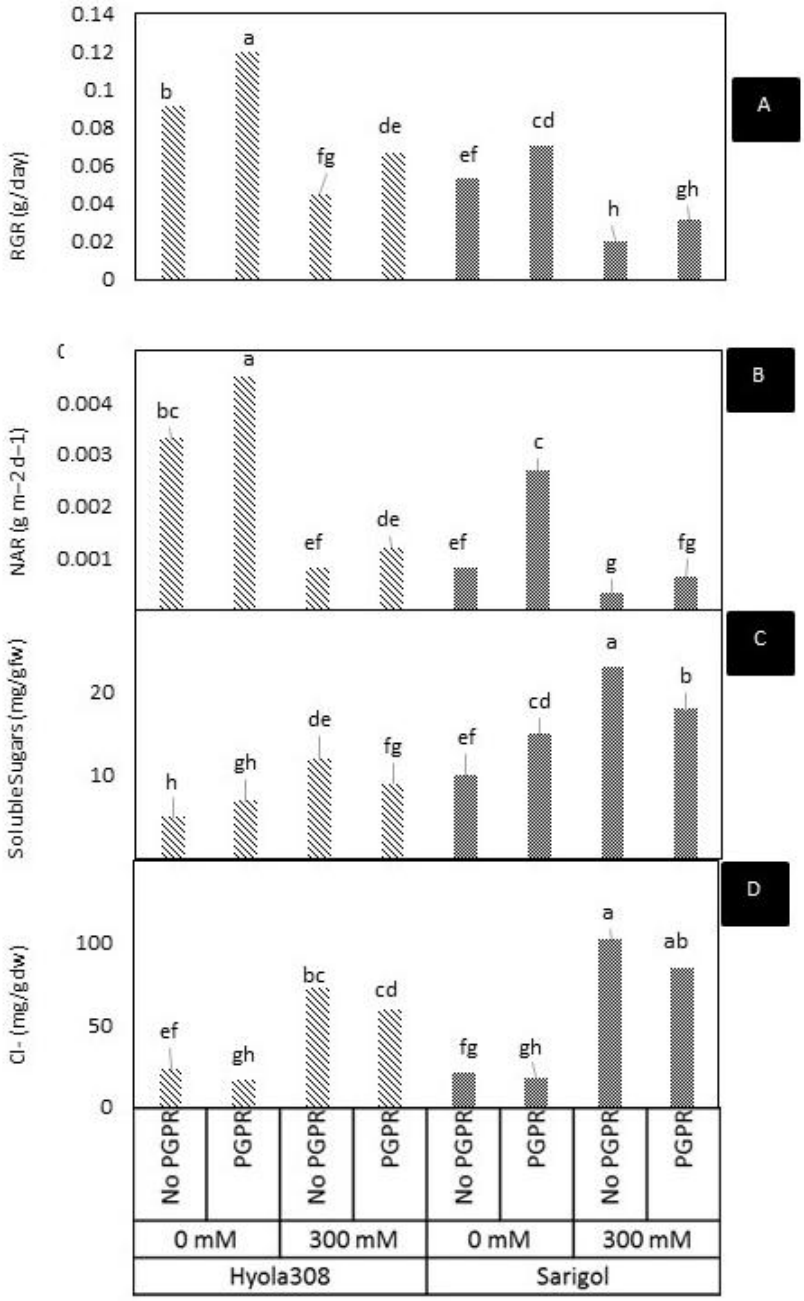


**Figure 1.** Physiochemical response of Salinity and bacterial inoculation on canola cultivars. comparing means between two tolerant (Hyola308) and sensitive (Sarigol) cultivars of canola under salinity (0 or 300 mM NaCl) and *Pseudomonas fluorescens* FY32 presence (it is implied as "PGPR") for relative growth rate (RGR) (**A**), net assimilation rate (NAR) (**B**), Soluble sugar contents in leaf (**C**), and chloride ion content in leaf (**D**) were performed. Same characters on the bars refer to non-significant differences.

**Table 1.** Analysis of variance for physiochemical characteristics of canola cultivars under salt stress and inoculated with P. fluorescens FY32.

| | | Mean square | | | |
|---|---|---|---|---|---|
| S.O.V | | RGR | NAR | S.S. | $Cl^-$ |
| Salinity | 1 | 0.019** | 0.0009** | 188.3** | 8674.7** |
| E1 | 2 | 0.0001 | 0.0001 | 1.6 | 175.4 |
| B | 1 | 0.001** | 0.004** | 120.9** | 978.4** |
| S× B | 1 | 0.0005 | 0.0008 | 4.7** | 65.7* |
| E2 | 4 | 0.0003 | 0.0003 | 0.045 | 6.8 |
| C | 1 | 0.0006* | 0.0005** | 258.4** | 1258.9** |
| S × C | 1 | 0.0003 | 0.000032 | 11.5** | 3816.1** |
| B × C | 1 | 0.0004 | 0.000012 | 8.3** | 122.9 |
| S × B × C | 1 | 0.0002 | 0.00002 | 0.53** | 29.7 |
| E3 | 8 | 0.0002 | 0.00006 | 0.049 | 27.1 |
| C.V. for Sub-Sub plots (%) | - | 18.54 | 21.49 | 1.95 | 10.73 |

Abbreviations: S.O.V, source of variance; B, Bacteria; S; salinity; C, Cultivar; E1, Main error; E2, Sub error; E3, Sub-sub error; d.f., degrees of freedom; RGR, Relative Growth Rate ; NAR, Net Assimilation Rate; S.S., Soluble Sugars; *, significant in 95% probability; **, significant in 99% probability.

***Soluble sugar and chloride ion contents in leaves:*** Salt tolerance in plants is a complex phenomenon. Organic solutes such as soluble sugars are helpful in osmoregulation and tolerance under toxicity of ions under salt stress [33]. In this study, soluble sugar content in leaves significantly decreased under salt stress. The effects of salt, bacterial inoculation, and 3-way interaction were significantly different for this trait (**Table 1**). Overall, Sarigol had a higher content of soluble sugars than Hyola308 (**Figure 1C**). The osmotic potentials of both root and leaf decreased under salinity, which was corroborated by the higher amounts of proline and soluble sugars [34]; however, in the present study, Sarigol accumulated more soluble sugars in leaf under salt stress. These results are consistent with previous studies [35]. Since soluble sugars may act like typical osmoprotectants, which stabilize cellular membranes and maintain turgor pressure [36], it could be claimed that Hyola308 triggers other pathways beyond utilizing soluble sugars to tolerate salt stress. The accumulation of soluble sugars in Sarigol was not as effective as triggering other pathways in Hyola308. In fact, tolerance is composed of various cross-talk among stress-response signaling pathways [37]. These results suggest that the high soluble sugar content in Sarigol leaves inevitably leads to dysfunction of this pathway and loss of plant tolerance to high salt stress. In this study, proteomic analysis was performed using tolerant and sensitive cultivars to clarify what other pathways are operative in Hyola308, which confer salt-stress tolerance.

## 3.2. Proteomic characterization

Two-week-old canola seedlings were transplanted into a hydroponic system and subjected to treatment with or without 300 mM NaCl and with or without bacterial inoculation. Leaf proteins were extracted and separated by isoelectric point/molecular weight using 2-DE. After CBB staining, the scanned gel images were analyzed using PDQuest software to evaluate the accumulation levels of proteins (**Figure 2**). In this

study, Mercator was used to identify the functional class of identified proteins using their acquired sequence. Mercator is a tool for classifying protein or gene sequences into functional plant categories in MapMan, many of which deal with metabolic pathways and enzyme functions.

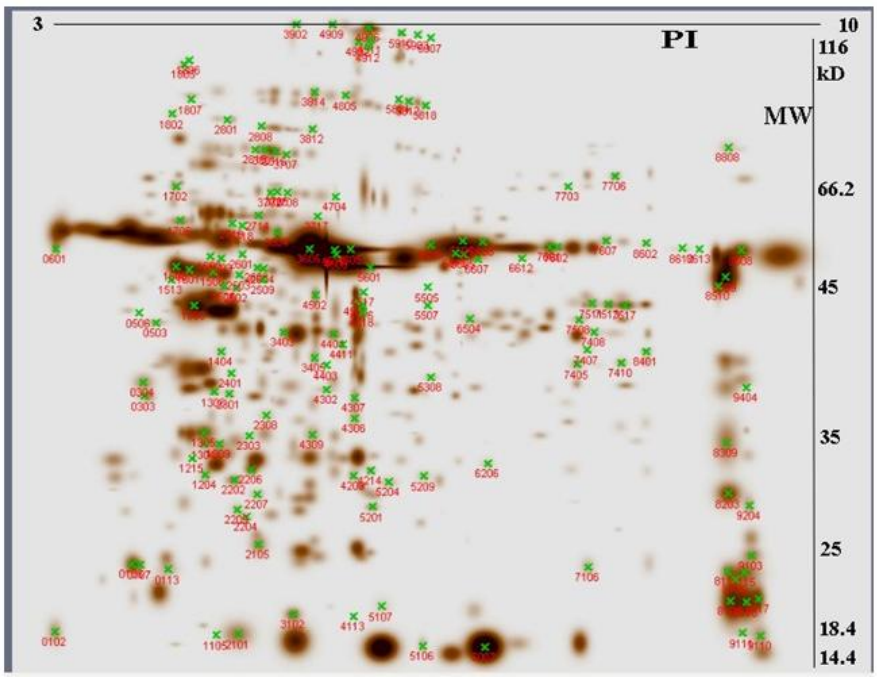


**Figure 2.** The Position and SSPs on master gel. An artificial software-constructed 2DE gel image based on all 2DE gels in the given experiment based on the quantification of spots on all gels, derived from PDQuest software. PI, Isoelectric point; MW, Molecular weight.

***Salt-stress responsive proteins:*** Analysis indicated that 78 protein spots were differentially abundant under salt stress and bacterial inoculation in Hyola308 (**Table S2**). Twelve proteins were identified as salt-stress responsive proteins, from which 8 proteins increased and 4 proteins decreased due to salt stress in Hyola308. In Sarigol, 62 proteins were differentially abundant under salt stress and inoculation conditions (**Table S3**), but 6 proteins were significantly identified to be salt-stress responsive proteins as all of 6 proteins increased due to salt stress. Classification of salt stress-responsive proteins into functional groups indicated that the regulation of proteins in tricarboxylic acid (TCA) cycle, amino acid metabolism, abiotic stress-related pathways, thioredoxin, RNA regulation/processing, protein degradation/targeting, and cell cycle/organization enhanced tolerance to salt stress (**Table S2**). The classification of salt-responsive proteins in both tolerant and sensitive cultivars indicates that the tolerant cultivars attempt to maintain the proper structure of RNA and proteins along with metabolism by re-regulating protein expression in related pathways. However, the sensitive cultivar under salt stress re-regulates the proteins related to photosynthesis/non-reductive PP transketolase, glycolysis, abiotic stress-related

proteins, nucleotide metabolism, and development. Previous studies using gel-free methods [15] support these results.

***Proteins changed in Hyola308 under salt stress:*** LSD1-like protein family (no 3504 in **Figure 3** and **Table S2**; no 4911 in **Table S3**), which accumulated 2-fold in Hyola308, is involved in biological processes such as histone H3-K4 methylation, histone deacetylation, and oxidation-reduction process, regulation of transcription, root development, transcription, and DNA-templated [38]. All 4 *Arabidopsis* LSD 1 proteins contain a flavin amine oxidase domain and a SWIRM domain, as well as being present in a number of proteins involved in chromatin regulation [39]; suggesting that the best prediction for this protein is "lysine-specific histone demethylase 1 homolog 2-like". Although research introduces various roles for this protein in mammals [40], these results indicate that LSD 1 expression levels may be affected by salt stress in canola. Although LSD 1 is involved in chromatin regulation, it might play a role in salt-stress tolerance in Hyola308, which is a tolerant cultivar.

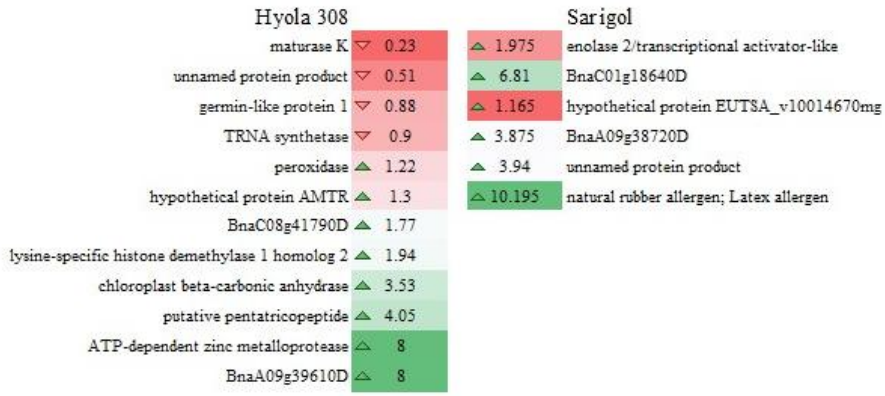


**Figure 3.** identified stress responsive proteins in two cultivars of canola. The proteins up-regulated (green) and proteins down-regulated (red) under saline condition were indicated.

As another important protein, that is affected by salt stress, ubiquitin-protein ligase (no 4909 in **Table S2**) increased under salt stress. Ubiquitination facilitates the growth and development of all eukaryotic species. In plant species, the ubiquitin 26S proteasome system has a role in the regulation of fundamental processes such as embryogenesis, photomorphogenesis, and organ development. The regulation of polyubiquitin-gene expression under salt stress indicated that the ubiquitin 26S proteasome system was involved in regulating plant-stress tolerance [41]. The transcription of *OsPUB* under various stress conditions indicates that the PUB gene might have various functions in the responses of rice to abiotic and biotic stresses [42]. Hyola308 as a tolerant cultivar increased this protein to tolerate the severe salinity condition.

Peroxidase superfamily (no 7703) increased under salt stress in Hyola308. Peroxidases, which are abiotic stress-inducible proteins and classify it into the group of stress-related proteins [43], are found in all plants as a family of different isozymes. They contain monomeric glycoproteins with heme structure, utilizing either $H_2O_2$ or $O_2$

to oxidize a wide variety of molecules. Based on the role of peroxidase genes under salt stress conditions, overexpression of the *TPX2*, and the *swpa1* genes resulted in increased salt tolerance. The growth-promoting effect of the peroxidase gene in transgenic tobacco is also reported [44]. The regulatory network comprising enzymatic and non-enzymatic antioxidant systems tends to keep the magnitude of ROS in plant cells to a non-damaging level. However, under salt stress, the production rate of ROS increases exponentially, exceeding the potential of antioxidant scavengers instigating oxidative burst, which affects biomolecules and disturbs cellular redox homeostasis [45]. To mitigate and repair damage initiated by ROS, plants have developed a complex antioxidant system [46]. In this study, the accumulation of peroxidase is due to the plant's effort to resist the salinity stress and reduce the destructive effects of ROS. Although some other studies claim that germin-like proteins increase under environmental stresses [47], a germin-like protein (no 6612 in **Table S2**) is decreased by 0.88 fold under salt stress in Hyola308 in this study (**Figure 3**). Results indicate that germin activity has a role in plant defense through the local production of $H_2O_2$ for the hypersensitive defense response [48]. The decrease of this enzyme in this study could be due to the success of other compensatory measures to eliminate hydrogen peroxide.

***Proteins changed in Sarigol under salt stress:*** Some energy/metabolism-related proteins were identified to play a role in salt stress in Sarigol. Two functional domains are recognized, which makes it a double functional protein with a role in photosynthesis (pyruvate-ferredoxin oxidoreductase domain) and transketolase activity (non-reductive PP transketolase domain) (no 4502 in **Table S3**). A glycolysis enzyme (enolase; no 206) decreased under salinity stress. Other proteomic approaches on various tissues of different plants have results that resemble this [49]. Enolase is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the ninth and penultimate step of glycolysis [50]. Probably energy availability or increased energy consumption due to stress rises from declined expression level of this enzyme in glycolysis [51].

On the other hand, a protein with Uracil phosphoribosyltransferase domain and an N-terminal uracil kinase domain put it into a classification of nucleotide metabolism (no 3504 in **Table S3**). The activity of this enzyme is increased under salt stress condition [52]. Since salt stress causes acceleration of purine catabolism [53], salt-treated plants probably attempt to balance the ratio of nucleotide degradation to synthesis, justifying the increased accumulation of this protein. Some other groups had responded to salinity stress in Sarigol. For instance, a hypothetical protein (no 1811 in **Table S3**) was identified as a germin-like protein, which is associated with abiotic stress in plants. Other hypothetical proteins (no 6502 and no 6703 in **Table S3**) were increased under salt stress, which is a development-storage protein with a role in seed filling. This is the same protein in protein (no 1710 in **Table S2**) in Hyola308, but it decreased in Hyola308; suggesting that this discrepancy is due to Sarigol being sensitive to saline conditions. Plants exposed to stress try to fill their seeds as soon as possible [54]. A tolerant plant could do this efficiently in a proper time while resisting the stress [55]. Storage proteins increase under severe environmental conditions [56] that resemble these results but it is recognized that a sensitive cultivar, Sarigol, is defective to fulfill this stage.

***Bacterial inoculation-responsive proteins:*** Thirty-seven proteins were identified as inoculation responsive proteins in Hyola308, in which 31 proteins increased and 6 proteins decreased due to bacterial inoculation. All proteins, which increased under salt stress responded to inoculation too in Hyola308 (**Table S2**, **Figures 4** and **5**). In Sarigol, 25 proteins were identified as inoculation responsive proteins as 21 proteins increased and 4 proteins decreased due to bacterial inoculation. Like Hyola308, all salt-responsive proteins in Sarigol increased due to inoculation (**Table S3** and **Figure 5**). PGPR induces various mechanisms that induce abiotic stress tolerance in plants. Mechanisms such as phosphate solubilization [57], nitrogen fixation [58], and siderophore production [59] have been shown to play a role in promoting plant growth by improving nutrient uptake. Moreover, it was shown that PGPR modulates the plant hormone status as one of their efficient mechanisms to improve plant tolerance under stress conditions by producing auxins and cytokinins [60] or by reducing plant ethylene levels via the bacterial enzyme ACC deaminase, which hydrolyses the ACC, immediate ethylene precursor, into α-ketobutyrate and ammonia [61]. In a molecular level, It seems that irrespective of mechanisms mediating plant-bacteria interaction, the basic mode of communication involves overall impacts on plant signaling pathways [62]. Against this background, it is useful to identify and summarize the relevant proteins in the aforementioned processes. The present results indicate that bacterial inoculation affects Hyola308 (**Figure 6A**) and Sarigol (**Figure 6B**) in various biological processes (**Figures 4** and **5**). All proteins identified to respond to salt stress in Hyola308 and Sarigol were changed by bacterial inoculation. This might arise from pathological effects of *Pseudomonas* on canola. In fact, *Pseudomonas* is an extracellular bacteria (ePGPR), existing in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex [63]. This can have minor stressful effects on the plant; however, the positive promoting effects of bacterial inoculation overcome pathogenic effects. The protein group contained in Hyola308 are proteins that affect plant metabolism, photosynthesis, glycolysis, DNA/RNA/protein metabolism, TCA cycle, secondary metabolite metabolism, hormone metabolism, signaling, gene expression regulation, stress, plant development (**Figure 6B**).

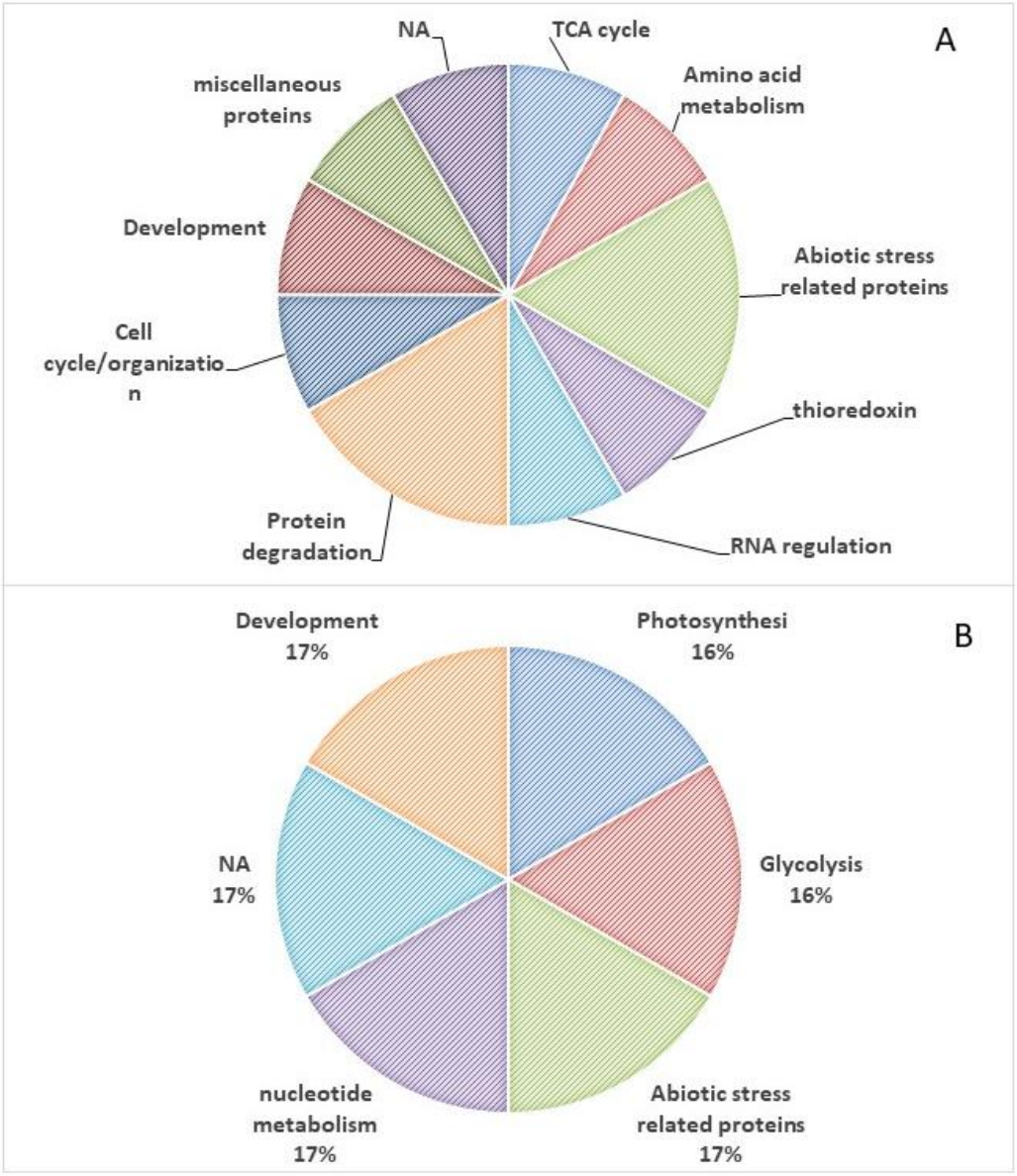


**Figure 4.** Functional classification of salt-responsive proteins and relative abundance of proteins into each group in Hyola308 (**A**) and Sarigol (**B**) cultivars. the data extracted from **Table S1**.


**Figure 5.** identified inoculation responsive proteins in two cultivars of canola. The proteins up-regulated (green) and proteins down-regulated (red) under saline condition were indicated.

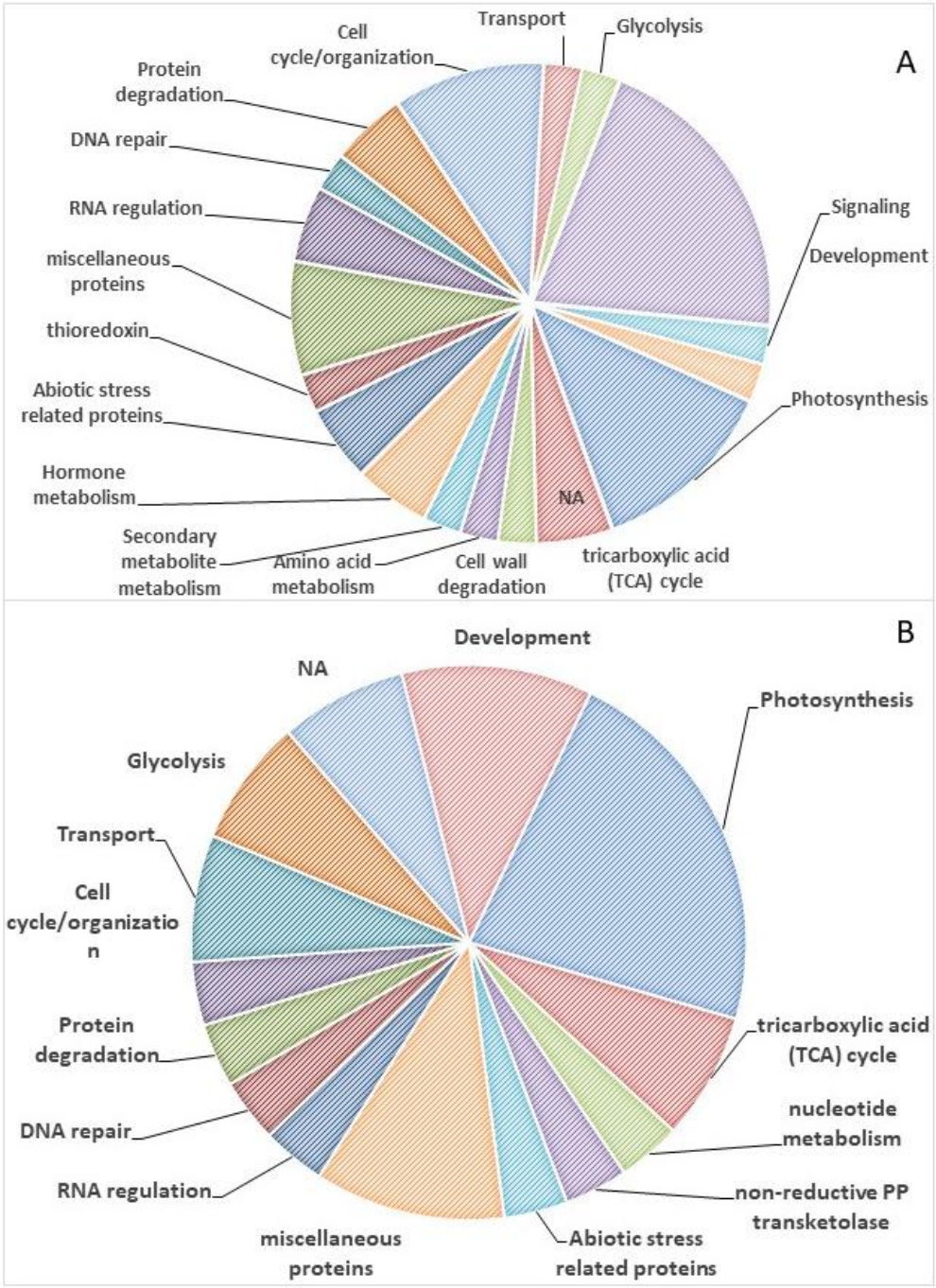


**Figure 6.** categorization of inoculation responsive proteins into functional groups and relative abundance of proteins into each group in Hyola308 (**A**) and Sarigol (**B**).

***Proteins changed in Hyola308 with bacterial inoculation:*** Bacterial inoculation on the root of Hyola308 has some effects on the accumulation of some photosynthetic (PS) proteins. Ribulose bisphosphate carboxylase, hypothetical protein VITISV, BnaA08g28710D, 33kDa oxygen-evolving protein, and BnaA08g28710D were identified as PS-related proteins (**Table S2**). The accumulation of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) activase, unlike other PS-related proteins, decreased under salt stress. Another study investigated the effect of moderate heat stress on the diurnal expression of 3 RuBisCO activase genes in cotton and found

that steady-state mRNA levels were reduced in the short term by heat stress [64], suggesting that mRNA degradation exceeds mRNA synthesis of this gene [65]. It is observed that bacteria inoculation has the same effect on the expression level of RuBisCO activase as heat. A hypothetical protein VITISV as L-ribulose-5-phosphate 3-epimerase, which increased after bacteria inoculation. This enzyme, as an isomerase, converts L-ribulose 5-phosphate to L-xylulose 5-phosphate in carbohydrate metabolic process. This reaction is in relation to the pentose phosphate pathway and is a common reaction of plants when they are exposed to PGPRs [66]. PS II subunits, which are oxygen-evolving enhancer (OEE) protein, is also known as a protein involved in detoxification of ROS and defense against bacteria (no 4807 and no 5708 in **Table S2**) [67]. The plant might recognize the bacteria as a pathogen and employ several mechanisms including OEEP for defense. On the other hand, the advantages of the presence of this PGPR are more than its pathogenic effects and lead the plant to more growth. An extrinsic subunit of PS II (no 5708), which plays a central role in stabilization of the catalytic manganese cluster [68]. These results suggest that this is one of those advantages of exposing canola to *P. fluorescens* FY32, which makes photosynthetic complexes more stable.

Two TCA-related proteins increased in the presence of PGPR. The lactate/malate dehydrogenase family protein was identified (no 503 in **Table S2**) and malate dehydrogenase (MDH) activity significantly changed in response to *P. fluorescens* treatments [69]. The TCA cycle is one of the most important decomposition energy acquisition pathways, and this pathway is affected by the activation of several key enzymes by PGPR, which may be the reason why plants are more resistant to salt stress than non-inoculated plants. The another protein (no 5507 in **Table S2**) is a carbonic anhydrase enzyme, which has responded to salt stress and inoculation. Carbonic anhydrase is a zinc-containing metalloenzyme, which catalyzes the reversible interconversion of $CO_2$ and $HCO^{3-}$. It plays an important role in many physiological functions, which involve decarboxylation or carboxylation reactions, including both photosynthesis and respiration. It also participates in the transport of inorganic carbon to actively photosynthesizing cells or away from actively respiring cells [70]. This protein increased under salt stress and bacterial inoculation in Hyola308. This can be considered as justification for the power of Hyola308 as a tolerant cultivar.

Certain PGPRs encompass an important enzyme such as 1-aminocyclopropane-1-carboxylate deaminase, which regulates ethylene synthesis by metabolizing ACC into α-ketobutyrate and ammonia [71]. *P. fluorescens* FY32 is characterized as an ACC deaminase encoding strain [13]. Inoculation with such PGPRs could be helpful in supporting plant growth and development under harsh environments by reducing stress-induced ethylene synthesis [72]. In this study, 2 hormone metabolism mediating proteins (no 7517 and no 7704 in **Table S2**) were identified in Hyola308 as responsive proteins to inoculation. An *N*-acetyltransferase protein (no 7517), which is involved in ethylene metabolism, may be another way that *P. fluorescens* stimulates plant growth in canola cultivars. On the other hand, myrosinase (no 7704) and its natural substrate, glucosinolate, are known to be a part of the plant-defense response [73]. When the plant is attacked by pathogens, insects, or another herbivore, the plant uses myrosinase to convert glucosinolates, which are otherwise-benign, into toxic products

like isothiocyanates, thiocyanates, and nitriles [74]. Regardless of whether *P. fluorescens* FY32 plays a role as a plant growth-promoting bacterium, we speculate that it also plays a role in recognizing this condition as an invasion on the plant side.

*P. fluorescens* FY32 has affected the level of 2 proteins in Hyola308, which have a role in protein targeting and degradation (no 2201 and no 3810 in **Table S2**). Importin beta domain family protein (no 2201) is involved in nuclear transport in abscisic acid (ABA) signaling, which is involved in a variety of plant growth and developmental processes [75]. It has been shown repeatedly that ethylene impacts on ABA biosynthesis and vice versa [76], and ABA might have an important role to make a plant resistant to environmental harsh conditions [44]. Our results suggest that increased levels of ethylene metabolism-related proteins reduce ABA levels in plants, and canola appears to try to compensate by reducing another protein (no 2201 in **Table S2**). FtsH is an ATP-dependent metalloprotease (no 3810 in **Table S2**) in thylakoid membranes as a hexameric heterocomplex, which increased in canola leaves with the presence of bacteria in the rhizosphere. A series of biochemical studies demonstrated that FtsH in thylakoids degrades photodamaged D1 reaction center proteins in PSII repair cycle [77]. Loss of several FtsHs results in leaf-variegated phenotypes in *Arabidopsis* [78]. This shows that canola tries to maintain its normal leaf condition and plastid functions under the condition of PGPR presence.

***Proteins changed in Sarigol with bacterial inoculation:*** Bacteria inoculation could affect the accumulation of some photosynthesis-related proteins in Sarigol (**Figure 6B**). One of the key proteins in this functional group is ATPase epsilon subunit (no 7108 in **Table S2**), which clearly functions as the main producer of ATP, utilizing the proton gradient generated by oxidative phosphorylation in mitochondria or photosynthesis in chloroplasts during cellular respiration. Subunit epsilon plays a key role in this regulation, affecting the efficiency of coupling, influencing the catalytic pathway and selectively inhibiting ATP hydrolysis activity [79]. According to these results, epsilon subunit has regulatory effects on energy production and has effects on plant growth and development [80]. Therefore, accumulation of this protein under bacterial inoculation may be another reason for the growth-promoting effect of this bacterium.

The catalytic subunit A of the vacuolar ATP synthase (no 3804 in **Table S3**) increased with the presence of PGPR in a sensitive cultivar of canola. Although different stresses induce the activity of this protein [81], *P. fluorescens* FY32 can induce up-regulation of subunit A of vacuolar ATPase. The vacuolar $H^+$-ATPase is a common component of eukaryotic creatures. It is present in the membranes of many organelles, where its proton-pumping action creates the low intra-vacuolar pH (e.g. in lysosomes). Both *N*-ethylmaleimide (NEM) and the nucleotide analogue 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride (NBD-Cl) are specific inhibitors of V-ATPase activity and both bind to subunit A in a manner that can be blocked by ATP in the presence of bivalent cations [82]. It is suggested to study these organic compounds and how their quantities are affected by the symbiotic relations in plants.

The most important enzyme complex in the TCA cycle, the pyruvate dehydrogenase complex, is composed of multiple subunits of 3 enzymes: decarboxylase, dihydrolipoyl acyltransferase, and lipoamide dehydrogenase. In this study, F21D18.28 (no 3708 in **Table S3**) is recognized as lipoamide dehydrogenase -

using Mercator tool—which has significantly up-regulated due to bacteria inoculation in Sarigol (**Figure 6B**); indicating that this enzyme has a tissue-specific and developmental-specific expression [83]. High accumulation of lipoamide dehydrogenase by PGPR could provide sufficient energy to leaves, resulting in high leaf area in the inoculated canola, which could be inferred from the proteomic analysis and biochemical characteristics of inoculated canola plants.

A member of FAD-binding Berberine family protein (no 2307 in **Table S3**) increased with the presence of P. *fluorescens* FY32 in the rhizosphere of sensitive cultivar of canola (**Table S3** and **Figure 5**). This family has electron carrier activity, oxidoreductase activity and FAD binding, catalytic activity. These family of proteins can play role in anthesis, petal differentiation and leaf expansion which might be helpful to early flowering and maturation of the plant [84–85]. Spot number 7706 in **Table S3** is predicted as ATP-dependent Clp protease (ATP-binding subunit/ClpC) with role in protein degradation in which that is involved in protein importation into the chloroplast and may provide ATP source that triggers TIC (Translocon at the Inner envelope membrane of Chloroplasts) translocation machinery [86]. Since its disruption prevents phototrophic growth in green algae [87], its function is apparently essential for chloroplasts. Incorrectly processed precursors imported from the cytosol are also degraded in the stroma by enzymes with the functional characteristics of Clp proteases [88]. As this protein increased in the presence of PGPR (**Figure 5**), this process may reduce the proportion of misfolded proteins when canola is inoculated with *P. fluorescens* FY32. These results indicate that other biochemical pathways can be carried out more efficiently, leading to better utilization of photosynthetic catalytic biochemicals and resulting in further plant growth.

## 4. Conclusions

Proteomic profiling of canola under different treatments has helped to identify systemic responses of the plant to multiple environmental stimuli, including salinity stress and plant growth promoting bacteria. In this study, the tolerant cultivar and/or using *P. fluorescens* FY32 as PGPR bacteria, had better performance based on the physiological and biochemical traits. Salt stress affected some proteins in both cultivars; the plants try to maintain their basic biological processes such as glycolysis, TCA cycle, and photosynthesis. The tolerant might had less energy consumption than Sarigol for launching the proper pathway/mechanism to tolerate salt condition. It can be viewed, that slat stress has main roles in the destruction of membranes, nutrient imbalance, impair the ability to detoxify ROS, differences in the antioxidant enzymes and decreased photosynthetic activity, and decrease in stomatal aperture followed by accumulation of $Na^+$ and $Cl^-$ ions in tissues of plants affects the related proteins in these processes. The groups of proteins that are affected by inoculation of *P. fluorescens* FY32, are similar to salt stress condition. Hence, it could be inferred that the presence of PGPR can regulate the tolerance mechanism by affecting the plants in the same way at which promotes growth. Here, transgenic studies at which genes related to growth regulation are manipulated would be helpful to understand the both salt-tolerance and plant-PGPR interaction mechanisms.

**Supplementary Materials**: Table S1. Protocol of gel-based proteomics; Table S2. Inoculation/Salinity-responsive proteins in Hyola308 leaves. Table S3. Inoculation/Salinity-responsive proteins in Sarigol leaves. Figure S1. Experimental design. Figure S2. 2D-PAGE patterns used in this research.

**Author contributions:** Conceptualization, AB and SK; methodology, FBA; software, FBA; investigation, EDU and DF; writing—original draft preparation, FBA; writing—review and editing, AB and SK; visualization, SK; supervision, AB and SK. All authors have read and agreed to the published version of the manuscript.

**Funding**: This work has been partially supported by Iran National Science Foundation (86121106).

**Data availability**: All data were mentioned in supplementary materials.

**Conflict of interest:** The authors have no conflict of interest.

**Ethics and consent to participate**: The authors declare that the Canola cultivars Hyola308 and Sarigol in this research were used and cultivation complied with national guidelines.

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**Supplementary materials file**

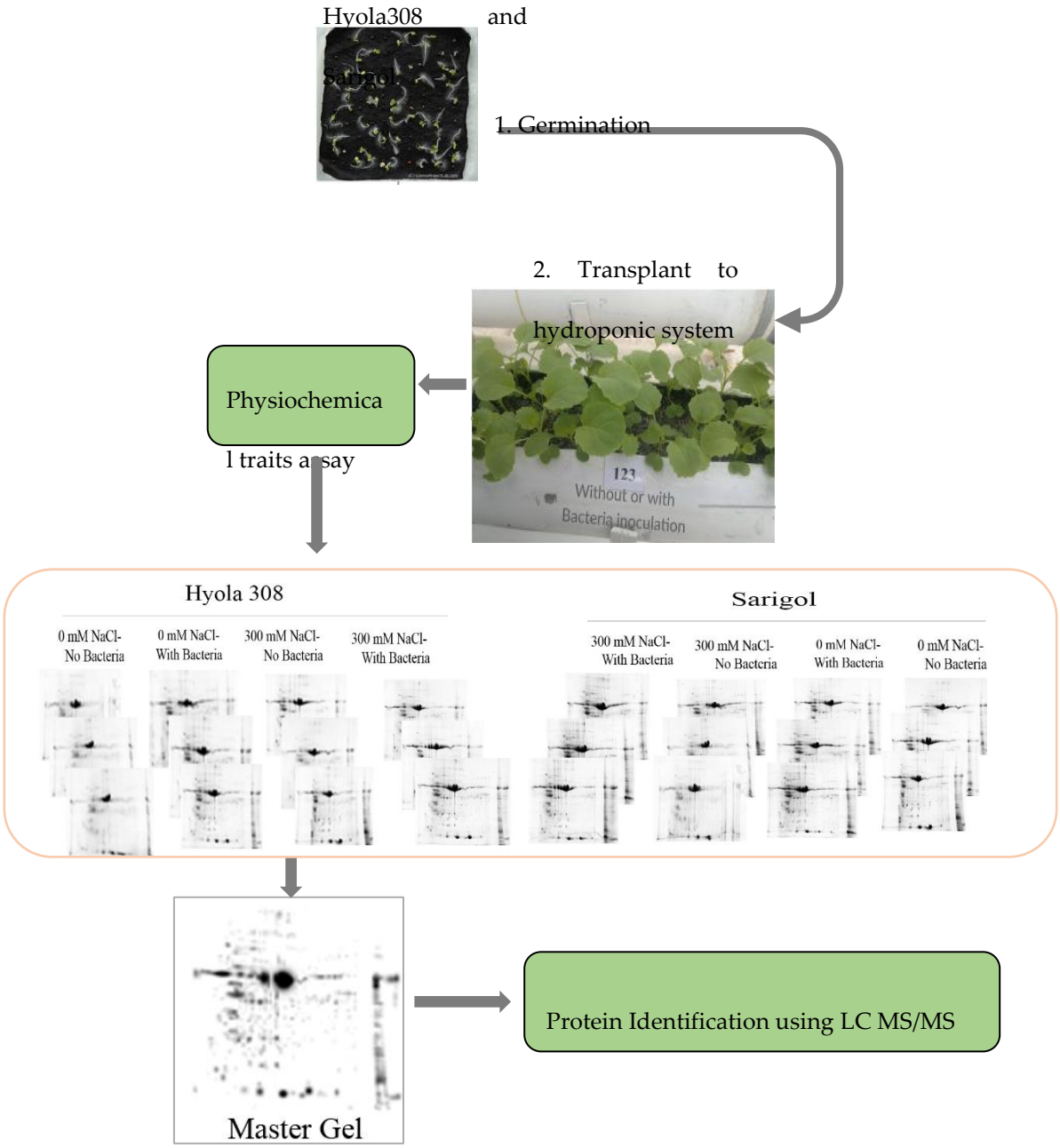


**Figure S1. Experimental design.**

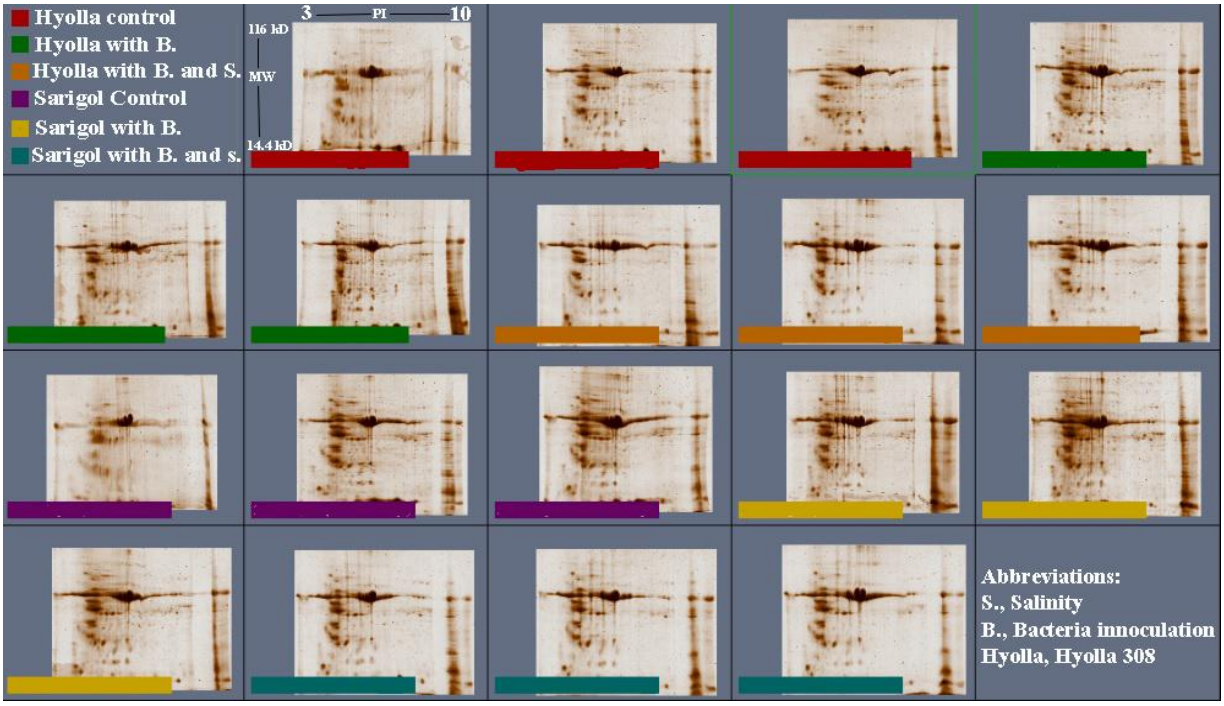


**Figure S2. 2d-page patterns used in this research.**

**Table S1**. Protocol of gel-based proteomics.

| Title | protocol |
|---|---|
| Two-dimensional polyacrylamide gel electrophoresis | Protein samples (400 µg) in a final volume of 180 µL lysis buffer containing 0.4% ampholyte (pH 3–10; Bio-Lyte, Bio-Rad, Hercules, CA, USA) were loaded into a focusing tray. Immobilized pH gradient strips (3–10 NL, 11 cm, Bio-Rad) were passively rehydrated for 2.5 h and then actively rehydrated for 14 h at 50 V. Isoelectric focusing (IEF) was carried out using a Protein IEF Cell system (Bio-Rad) under the following conditions: 250 V for 15 min with a linear ramp, 8000 V for 1 h with a linear ramp, and finally 8000 V for 35,000 V-h with a rapid ramp. After IEF, the strips were incubated for 15 min in equilibration buffer I consisting of 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol, and 130 mM dithiothreitol. The strips were incubated for 15 min in equilibration buffer II, consisting of 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol, and 135 mM iodoacetamide. The equilibrated strips were placed onto 15% SDS-polyacrylamide gel electrophoresis gels and sealed with 1% low-melting-temperature agarose (Komatsu et al., 2010). The second dimension of electrophoresis was performed at a constant current of 30 mA. After electrophoresis, the gels were stained for 1 h in a solution of Coomassie brilliant blue (CBB) (PhastGel™Blue R, GE Healthcare, Piscataway, NJ, USA) containing 35% methanol and 10% acetic acid, and then de-stained for 12 h in a solution of 35% methanol and 10% acetic acid. |
| Preparation of proteins for mass spectrometry | In order for gel-based proteomic analysis, spots were cut-off from the CBB-stained gels and washed with water. Proteins in the excised gel pieces were reduced with 10 mM dithiothreitol in 100 mM $NH_4HCO_3$ for 1 h at 60 °C, and then further alkylated with 40 mM iodoacetamide in 100 mM $NH_4HCO_3$ for 30 min. The gel pieces were then digested in 100 mM $NH_4HCO_3$ with 1 pM trypsin (Wako, Osaka, Japan) at 37 °C overnight. The tryptic peptides were extracted from the gel matrix 3 times with 0.1% trifluoroacetic acid in 50% acetonitrile. The protein excision and extraction procedure were performed using the Digest Pro System (Intavis Bioanalytical Instruments AG, Cologne, Germany). The resulting peptide sample solutions were desalted with SPE C-Tip pipet tips (NikkyoTechnos, Tokyo, Japan), dried, and then mixed with 0.1% formic acid. The prepared samples were then analyzed by nano-liquid chromatography (LC) mass spectrometry (MS)/MS. |
| Data acquisition by nano-liquid chromatography-mass spectrometry for gel-based proteomics analysis | An Ultimate 3000 nano-LC system (Dionex, Germering, Germany) equipped with C18 PepMap trap column (300 µm ID × 5 mm, Dionex) was utilized for loading Peptides in 0.1% formic acid. The peptides were eluted from the trap column and were then separated on a C18 Tip column (75 µm 1D × 120 mm, NTTC-360/75-3 nano-LC capillary column; NikkyoTechnos) and analyzed on a nanospray LTQ XL Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA) based on the method presented by Banaei-Asl et al (Banaei-Asl et al. 2015). The analysis was carried out using Xcalibur software (version 2.0.7; Thermo Fisher Scientific). Full-scan mass spectra were acquired in the Orbitrap MS over 400–1500 m/z with a |

resolution of 30,000. The 3 most intense ions above the 1000 threshold value were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35% after accumulation to a target value of 1000. To prevent the reiterative selection of peptides, dynamic exclusion was recruited within 30 s. the attained spectra were applied for protein identification.

The obtained MS and MS/MS spectra were used to identify differentially abundant canola proteins using the MASCOT search engine (version 2.4.1; Matrix Science, London, UK) and Protein Discoverer (version 1.4.0.288, Thermo Fisher Scientific) against Arabidopsis peptide database (TAIR10, http://www.arabidopsis.org/; 35,386 entries). In the MASCOT search, carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was acceptable. Peptide mass tolerance, 5 ppm; fragment mass tolerance, 0.5 Da; the maximum number of missed cleavages, 1; and peptide charges, +2, +3, and +4 were the parameters for search. The negligible obligation for accepting an identified protein (a) the score indicated the probability of a true positive identification, which at least was 81; (b) at least two peptide sequences matched above the identity threshold; and (c) the coverage of the total protein sequence by the matching peptides was more than 4%.

**Table S2**. Inoculation/Salinity-responsive proteins in Hyola308 leaves.

| | Ratio | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Salinity | | Bacteria Inoculation | | Protein identification data | | | | | | |
| SSP | 0 mM | 300 mM | Non-Inoculated | Inoculated | Description | GI Number | Function | Score | Mass | Num. of significant matches | P value |
| 1516 | 1 | ns | 1 | 3.3 | N/A | | | | | | 0.01 |
| 1401 | 1 | 0.145 | 1 | 1.45 | N/A | | | | | | 0.04 |
| 1302 | 1 | 8 | 1 | 8 | N/A | | | | | | 0.01 |
| 107 | 1 | 1.41 | 1 | 1.26 | N/A | | | | | | 0.02 |
| 3102 | 1 | 1.1 | 1 | ns | N/A | | | | | | 0.03 |
| 3205 | 1 | 4.52 | 1 | 0.16 | N/A | | | | | | 0.01 |
| 2209 | 1 | 4.47 | 1 | 1.3 | N/A | | | | | | 0.01 |
| 5910 | 1 | ns | 1 | 1.26 | N/A | | | | | | 0.01 |
| 5819 | 1 | ns | 1 | 1.3 | TCP2 | gi\|23307807 | RNA | 17 | 29637 | 1 | 0.04 |
| 5824 | 1 | ns | 1 | 0.53 | Ribulose bisphosphate carboxylase | gi\|266893 | PS | 67 | 45909 | 1 | 0.02 |
| 4805 | 1 | 0.8 | 1 | ns | N/A | | | | | | 0 |
| 3505 | 1 | 1.94 | 1 | 0.49 | lysine-specific histone demethylase 1 homolog 2 | gi\|514793679 | misc | 15 | 84512 | 1 | 0.02 |
| 7513 | 1 | 0.34 | 1 | ns | N/A | | | | | | 0.02 |
| 7517 | 1 | ns | 1 | 1.09 | probable N-acetyltransferase HLS1 | gi\|698573977 | Hormone. Met/misc | 17 | 45190 | 1 | 0.01 |
| 3709 | 1 | 1.02 | 1 | ns | N/A | | | | | | 0.01 |
| 4909 | 1 | 1.3 | 1 | ns | hypothetical protein AMTR | gi\|586776492 | Protein | 31 | 417734 | 1 | 0.03 |
| 3602 | 1 | ns | 1 | 2.6 | hypothetical protein MICPUCDRAFT | gi\|303288958 | Not Assigned | 23 | 31785 | 1 | 0.01 |
| 1215 | 1 | ns | 1 | 3.7 | N/A | | | | | | 0.04 |
| 2201 | 1 | ns | 1 | 0.51 | predicted protein | gi\|255089074 | Protein | 18 | 120659 | 1 | 0.04 |
| 8403 | 1 | ns | 1 | 8 | N/A | | | | | | 0.04 |
| 1710 | 1 | 0.51 | 1 | 0.4 | unnamed protein product | gi\|18609 | Development | 149 | 54869 | 4 | 0.01 |
| 2714 | 1 | ns | 1 | 3.7 | N/A | | | | | | 0.03 |
| 4214 | 1 | 0.41 | 1 | 0.43 | N/A | | | | | | 0.02 |
| 3712 | 1 | ns | 1 | 2.9 | BnaA04g25390D | gi\|674902579 | AA Met. | 421 | 41659 | 17 | 0.03 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 4405 | 1 | ns | 1 | 7 | hypothetical protein POPTR | gi\|224145982 | Signaling | 18 | 60702 | 1 | 0.05 |
| 5209 | 1 | ns | 1 | 8 | uncharacterized protein | gi\|698506271 | Not Assigned | 25 | 33836 | 1 | 0 |
| 2601 | 1 | ns | 1 | 0.09 | unnamed protein product | gi\|661882355 | Not Assigned | 33 | 43936 | 1 | 0.02 |
| 2207 | 1 | 0.57 | 1 | ns | N/A | | | | | | 0.04 |
| 503 | 1 | ns | 1 | 4.2 | Malate dehydrogenase | gi\|11133373 | TCA | 52 | 35866 | 1 | 0.04 |
| 1807 | 1 | 0.97 | 1 | ns | N/A | | | | | | 0.01 |
| 3812 | 1 | 8 | 1 | 8 | N/A | | | | | | 0.01 |
| 7703 | 1 | 1.22 | 1 | 1.75 | peroxidase | gi\|226532578 | Stress | 22 | 37880 | 1 | 0.05 |
| 5507 | 1 | 3.53 | 1 | 3.16 | chloroplast beta-carbonic anhydrase | gi\|297787439 | TCA | 633 | 36127 | 23 | 0.02 |
| 6612 | 1 | 0.88 | 1 | 1.53 | germin-like protein 1 | gi\|685270849 | Stress | 405 | 22052 | 14 | 0.05 |
| 5705 | 1 | ns | 1 | 9.4 | hypothetical protein VITISV | gi\|147817756 | PS | 159 | 33877 | 3 | 0.02 |
| 203 | 1 | ns | 1 | 8.16 | BnaA09g39610D | gi\|674921737 | Cell | 239 | 28223 | 5 | 0 |
| 204 | 1 | 4.05 | 1 | 1.81 | putative pentatricopeptide | gi\|720090598 | Not Assigned | 14 | 22528 | 1 | 0.05 |
| 1103 | 1 | 0.67 | 1 | ns | N/A | | | | | | 0.02 |
| 1311 | 1 | 1.89 | 1 | ns | N/A | | | | | | 0.02 |
| 1701 | 1 | 0.9 | 1 | 0.42 | TRNA synthetase | gi\|108864571 | Redox | 17 | 39741 | 1 | 0 |
| 1715 | 1 | ns | 1 | 1.02 | N/A | | | | | | 0 |
| 1716 | 1 | ns | 1 | 1.23 | N/A | | | | | | 0 |
| 1718 | 1 | 8 | 1 | 1.3 | N/A | | | | | | 0 |
| 1719 | 1 | ns | 1 | 7.3 | N/A | | | | | | 0 |
| 1811 | 1 | ns | 1 | 1.3 | stress response protein nst1-like | gi\|356538162 | Not Assigned | 114 | 82003 | 2 | 0.04 |
| 1813 | 1 | 1.5 | 1 | ns | N/A | | | | | | 0 |
| 2302 | 1 | 8 | 1 | 1.7 | BnaA09g39610D | gi\|674921737 | Cell | 184 | 28223 | 4 | 0.03 |
| 2403 | 1 | ns | 1 | 1.27 | N/A | | | | | | 0.01 |
| 2406 | 1 | ns | 1 | 8 | predicted protein | gi\|255081572 | DNA | 20 | 83648 | 1 | 0.05 |
| 2506 | 1 | ns | 1 | 10.49 | N/A | | | | | | 0.01 |
| 2701 | 1 | 0.13 | 1 | ns | N/A | | | | | | 0 |
| 2707 | 1 | 3.67 | 1 | ns | N/A | | | | | | 0.03 |
| 2720 | 1 | ns | 1 | 8 | N/A | | | | | | 0 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 3202 | 1 | ns | 1 | 5.4 | N/A | | | | | 0 |
| 3210 | 1 | ns | 1 | 1.1 | vacuolar amino acid transporter 1-like | gi\|698551908 | AA Transport | 15 | 60889 | 1 | 0 |
| 3302 | 1 | ns | 1 | 0.86 | N/A | | | | | | 0.04 |
| 3809 | 1 | ns | 1 | 8 | BnaA09g39610D | gi\|674921737 | Cell | 149 | 28223 | 3 | 0.01 |
| 3810 | 1 | 8 | 1 | 8 | ATP-dependent zinc metalloprotease | gi\|747099846 | Protein | 17 | 73345 | 1 | 0.05 |
| 3903 | 1 | ns | 1 | 0.53 | N/A | | | | | | 0.03 |
| 3904 | 1 | ns | 1 | 5.18 | N/A | | | | | | 0.03 |
| 4401 | 1 | ns | 1 | 2.4 | N/A | | | | | | 0 |
| 4408 | 1 | ns | 1 | 3.6 | transmembrane domain-containing protein | gi\|727604026 | Not Assigned | 20 | 113478 | 1 | 0.01 |
| 4807 | 1 | ns | 1 | 1.6 | BnaA08g28710D | gi\|674917727 | PS | 162 | 28142 | 8 | 0.01 |
| 4808 | 1 | ns | 1 | 1.1 | predicted protein | gi\|326490585 | Not Assigned | 14 | 9159 | 1 | 0 |
| 4809 | 1 | 5.87 | 1 | ns | N/A | | | | | | 0 |
| 5708 | 1 | ns | 1 | 5.9 | 33 kDa oxygen-evolving protein | gi\|22571 | PS | 44 | 35285 | 1 | 0.01 |
| 6201 | 1 | ns | 1 | 5.5 | fructose-bisphosphate aldolase | gi\|619835266 | Glycolysis | 404 | 38743 | 9 | 0.05 |
| 6202 | 1 | ns | 1 | 4.1 | N/A | | | | | | 0.04 |
| 6501 | 1 | ns | 1 | 6.25 | BnaA08g28710D | gi\|674917727 | PS | 136 | 28142 | 4 | 0.05 |
| 6701 | 1 | 6.1 | 1 | ns | N/A | | | | | | 0 |
| 6704 | 1 | 0.23 | 1 | 0.59 | maturase K | gi\|157689204 | RNA | 20 | 60816 | 1 | 0 |
| 6707 | 1 | ns | 1 | 5.7 | hypothetical protein PHAVU | gi\|593692546 | Not Assigned | 32 | 41158 | 1 | 0.02 |
| 7206 | 1 | 2.11 | 1 | ns | N/A | | | | | | 0.02 |
| 7701 | 1 | 1.77 | 1 | 1.71 | BnaC08g41790D | gi\|674898258 | AA Met. | 466 | 50409 | 15 | 0.04 |
| 7704 | 1 | ns | 1 | 2.1 | myrosinase-like | gi\|685381261 | Sec. Met./Hormone Met./misc | 618 | 62547 | 17 | 0.02 |
| 7705 | 1 | 8.34 | 1 | ns | N/A | | | | | | 0.02 |
| 8207 | 1 | ns | 1 | 8 | BnaA02g05160D | gi\|674924032 | Cell Wall | 558 | 69357 | 12 | 0.04 |
| 8402 | 1 | ns | 1 | 1.74 | BnaA09g39610D | gi\|674921737 | Cell | 121 | 28223 | 2 | 0.01 |

Abbreviations: PS, Photosynthesis; TCA, tricarboxylic acid (TCA) cycle; Cell Wall, Cell wall degradation; AA Met., Amino acid metabolism; Secondary Met., Secondary metabolite metabolism; Hormone Met., Hormone metabolism; Stress, Abiotic stress related proteins; Redox, thioredoxin; misc., miscellaneous proteins; RNA, RNA regulation/processing; DNA, DNA repair; Protein, Protein degradation/targeting; Cell, Cell cycle/organization; AA transport, Amino acid transport; SSP, sample spot protein. Significant threshold $p<0.05$ for protein identification.

**Table S3**. Inoculation/Salinity-responsive proteins in Sarigol leaves.

| | **Ratio** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Salinity | | Bacteria Inoculation | | | Protein Identification Data | | | | | |
| SSP | 0 mM | 300 mM | Non-Inoculated | Inoculated | Description | GI Number | Function | Score | Mass | Num. of significant matches | P value |
| 2408 | 1 | 5.075 | 1 | 4.75 | N/A | | | | | | 0 |
| 1215 | 1 | ns | 1 | 2.74 | N/A | | | | | | 0.02 |
| 4404 | 1 | ns | 1 | 3.19 | N/A | | | | | | 0.01 |
| 1506 | 1 | ns | 1 | 2.18 | N/A | | | | | | 0.05 |
| 1513 | 1 | ns | 1 | 2.52 | peptidyl-prolyl cis-trans isomerase | gi\|727475028 | Cell | 477 | 28974 | 22 | 0.02 |
| 1205 | 1 | ns | 1 | 1.215 | N/A | | | | | | 0.05 |
| 2815 | 1 | 1.55 | 1 | 0.285 | N/A | | | | | | 0.04 |
| 4902 | 1 | ns | 1 | 4.93 | N/A | | | | | | 0.01 |
| 1810 | 1 | 8 | 1 | 8 | N/A | | | | | | 0.05 |
| 3102 | 1 | 8 | 1 | 8 | N/A | | | | | | 0.04 |
| 1208 | 1 | ns | 1 | 0.905 | BnaC04g05700D | gi\|674912669 | PS | 326 | 51594 | 7 | 0.01 |
| 1718 | 1 | 0.2 | 1 | 0.215 | N/A | | | | | | 0.04 |
| 5201 | 1 | ns | 1 | 2.83 | N/a | | | | | | 0.05 |
| 2307 | 1 | ns | 1 | 2.4 | cannabidiolic acid synthase-like 2 | gi\|685358529 | misc | 23 | 60995 | 1 | 0.01 |
| 3406 | 1 | 8 | 1 | ns | N/A | | | | | | 0.01 |
| 7704 | 1 | ns | 1 | 7.1 | N/A | | | | | | 0.05 |
| 7405 | 1 | 0.275 | 1 | 8 | N/A | | | | | | 0.01 |
| 7706 | 1 | ns | 1 | 5.3 | chaperone protein ClpC1 | gi\|685358529 | Misc/Protein | 23 | 60995 | 1 | 0.02 |
| 1204 | 1 | 7.1 | 1 | ns | N/A | | | | | | 0.01 |
| 3717 | 1 | 3.5 | 1 | ns | N/A | | | | | | 0.03 |
| 1808 | 1 | ns | 1 | 4.59 | archaeal/vacuolar-type H+-ATPase subunit A | gi\|657386133 | Transport | 546 | 68949 | 15 | 0.04 |
| 118 | 1 | 5.155 | 1 | 8.49 | N/A | | | | | | 0.01 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4517 | 1 | ns | 1 | 1.865 | N/A | | | | | | 0.04 |
| 1603 | 1 | ns | 1 | 8 | hypothetical protein ZEAMMB73_828091 | gi\|413926649 | DNA | 17 | 210536 | 1 | 0 |
| 2606 | 1 | ns | 1 | 0.55 | BnaC03g60280D | gi\|674921158 | PS | 1017 | 41479 | 36 | 0.02 |
| 3709 | 1 | ns | 1 | 4.86 | N/A | | | | | | 0.01 |
| 3705 | 1 | ns | 1 | 8 | N/A | | | | | | 0.01 |
| 2105 | 1 | 6.6 | 1 | ns | N/A | | | | | | 0 |
| 3804 | 1 | ns | 1 | 8 | V-type proton ATPase catalytic subunit A | gi\|685333963 | Transport | 1386 | 68969 | 38 | 0.01 |
| 2503 | 1 | 2.13 | 1 | ns | N/A | | | | | | 0.03 |
| 7108 | 1 | ns | 1 | 1.785 | ATP synthase CF1 epsilon subunit | gi\|139387258 | PS | 600 | 14616 | 14 | 0.05 |
| 4502 | 1 | 2.33 | 1 | 3.875 | BnaA09g38720D | gi\|674891569 | PS/OPP | 362 | 79864 | 9 | 0.05 |
| 3809 | 1 | 0.87 | 1 | ns | N/A | | | | | | 0.04 |
| 3803 | 1 | 1.255 | 1 | 8.9 | N/A | | | | | | 0.01 |
| 3201 | 1 | ns | 1 | 0.275 | N/A | | | | | | 0.03 |
| 3714 | 1 | ns | 1 | 6 | N/A | | | | | | 0.01 |
| 206 | 1 | 0.085 | 1 | 1.975 | enolase 2/transcriptional activator-like | gi\|685305689 | Glycolysis | 756 | 47841 | 17 | 0.05 |
| 1302 | 1 | 2.01 | 1 | ns | N/A | | | | | | 0 |
| 4906 | 1 | ns | 1 | 4.055 | N/A | | | | | | 0.01 |
| 7509 | 1 | ns | 1 | 2.62 | N/A | | | | | | 0 |
| 1716 | 1 | ns | 1 | 1.275 | N/A | | | | | | 0 |
| 5504 | 1 | ns | 1 | 8 | N/A | | | | | | 0 |
| 204 | 1 | ns | 1 | 0.22 | Los (enolase) | gi\|90194338 | Glycolysis | 295 | 47812 | 4 | 0.01 |
| 1308 | 1 | ns | 1 | 5.78 | chloroplast Rubisco | gi\|471178967 | PS | 843 | 52209 | 19 | 0 |
| 1501 | 1 | ns | 1 | 3.705 | BnaC04g46560D | gi\|674940110 | PS | 424 | 48296 | 10 | 0.01 |
| 1805 | 1 | 0.65 | 1 | ns | N/A | | | | | | 0.01 |
| 1806 | 1 | ns | 1 | 6.98 | N/A | | | | | | 0 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1807 | 1 | ns | 1 | 8 | hypothetical protein M569_06084, partial | gi\|527201642 | Not Assigned | 19 | 56662 | 1 | 0.03 |
| 1811 | 1 | 1.945 | 1 | 1.165 | hypothetical protein EUTSA_v10014670mg | gi\|567176075 | Stress | 374 | 22085 | 10 | 0 |
| 2406 | 1 | ns | 1 | 7.31 | NADP-dependent alkenal reductase P1 | gi\|685278784 | misc | 276 | 38221 | 6 | 0.02 |
| 2604 | 1 | ns | 1 | 8.6 | N/A | | | | | | 0.01 |
| 2610 | 1 | ns | 1 | 2.52 | malate dehydrogenase | gi\|433335660 | TCA | 151 | 36038 | 4 | 0.01 |
| 3504 | 1 | 1.2 | 1 | 6.81 | BnaC01g18640D | gi\|674938986 | Nt. Met. | 287 | 96988 | 6 | 0.05 |
| 3708 | 1 | ns | 1 | 3.35 | F21D18.28 | gi\|297852420 | TCA | 176 | 54035 | 4 | 0.01 |
| 3902 | 1 | ns | 1 | 4.2 | B-Conglycinin with N-Acetyl-D-Glucosamine | gi\|21465628 | Developme nt | 117 | 47879 | 3 | 0.03 |
| 4911 | 1 | ns | 1 | 0.01 | unnamed protein product | gi\|18609 | Developme nt | 149 | 54869 | 4 | 0.02 |
| 5812 | 1 | ns | 1 | 3.77 | unknown | gi\|388508864 | RNA | 49 | 33580 | 1 | 0 |
| 6501 | 1 | 0.23 | 1 | ns | N/A | | | | | | 0.04 |
| 6502 | 1 | 5.935 | 1 | 10.195 | natural rubber allergen; Latex allergen | gi\|14423933 | Not Assigned | 25 | 22331 | 1 | 0.02 |
| 6703 | 1 | 2.795 | 1 | 3.94 | unnamed protein product | gi\|18609 | Developme nt | 112 | 54869 | 6 | 0 |
| 6707 | 1 | ns | 1 | 1.735 | N/A | | | | | | 0.02 |
| 7302 | 1 | ns | 1 | 2.175 | N/A | | | | | | 0 |

Abbreviations: PS, Photosynthesis; OPP, non-reductive PP transketolase; TCA, tricarboxylic acid (TCA) cycle; Stress, Abiotic stress related proteins; Nt. Met, nucleotide metabolism; misc., miscellaneous proteins; RNA, RNA binding proteins; DNA, DNA synthesis/chromatin structure; Protein, Protein degradation; Cell, Cell cycle; Transport, P and V ATPase transporter family. Significant threshold $p < 0.05$ for protein identification; SSP, sample spot protein