Journal of Applied Botany and Food Quality 98, 29 - 40 (2025), DOI:10.5073/JABFQ.2025.098.004

Department of Molecular Biology and Genetics, T.C. Istanbul Kültür University, İstanbul, Türkiye

Two dimensional proteomic analyses revealed salinity and drought tolerance related protein alterations in two gamma-induced soybean mutants

Alp Ayan*, Sinan Meriç, Tamer Gümüş, Özge Çelik, Çimen Atak

(Submitted: April 2, 2024; Accepted: February 1, 2025)

Summary

Soybean [*Glycine max* (L.) Merr], is an important industrial oil seed plant. Along with the nutritional value for humans and animals, it has raw materials for various industrial products. In the present study, we investigated the two-dimensional protein expression profiles in salinity and drought tolerant mutant plants derived from S04-05 soybean variety by Cs-137 gamma radiation source induced mutations. Altogether 54 different protein spot alterations were identified as salinity and drought responsive by two-dimensional electrophoresis and MALDI-TOF-MS. A protein-protein interaction network was constructed considering significantly altered proteins by STRING analysis software. Identified proteins, which presented differential expressions under both stress conditions, were clustered under 13 distinct groups based on their cellular functions. Two of these biological processes, photosynthesis and carbohydrate metabolism, were found significantly altered by KEGG analysis. Our results contribute proteomic data to salinity and drought tolerance of our soybean mutants, which originated from S04-05, a variety mildly susceptible to salinity and drought. These results may provide a basis for future investigations into the genetic and physiological aspects of both stress tolerances.

Keywords: *Glycine max* (L.) Merr; salinity tolerance; drought tolerance; mutation breeding; proteomics, Cs-137

Introduction

Soybean (*Glycine max* (L.) Merr) is an important nutrition for human diet due to its high protein and oil content. It is also used as a raw material for the animal feed industry. Soybean, which belongs to *Fabaceae* family, is an annual, dicot, multi-branched legume plant with 1.5-meter average length. Due to its high oil content as 12% palmitic acid, 4% stearic acid, 23% oleic acid, 53% linoleic acid and 8% linolenic acid, it is defined as an oil seed plant. Moreover, it provides raw materials to dough products, baby food, candy products, anti-allergic milk products, soy meat and more (Bellaloui, 2012). According to the 2018 harvested area (ha) statistics of Food and Agriculture Organization of the United Nations (FAO), USA, Brazil, Argentina, India, and China are the top producers, whereas in yield (hg/ha) statistics, Türkiye, Italy, USA, Georgia and Brazil are the leading countries (FAO, 2020). Despite the higher harvest areas some countries don't represent the same accomplishment in yield stats. This situation is mostly caused by factors such as limited genetic sources, abiotic stress factors, inaccurate agriculture applications and diseases (Fang et al., 2024).

Plants are subjected to various biotic and abiotic stress factors constantly due to their sessile nature. As they are subjected to these abiotic stress factors like drought, salinity, heavy metals, intensive light, high and low temperatures, inadequate nutrition, they alter their metabolic processes as a response. Salinity is described as accumulation of soluble salts on the upper layer of the soil as it surpluses 4 $dS.m^{-1}$ electrical conductivity (EC) value. Salinity, which is one of the most influential abiotic stress factors affecting productivity and quality in semi-arid and arid regions, causes reduction in water uptake, transpiration rate, photosynthesis, nitrogen assimilation, accumulation of growth inhibitors, degradation of biomolecules, alterations in gene expression and protein synthesis, losses in enzymatic activity, disruption of membrane systems. Also, when the duration and/or intensity of salinity persists, it leads to plant death and loss of yield (Hirt and Shinozaki, 2004; Parida and Das, 2005; Rao et al., 2006).

Long-lasting and systematic water deficiency is referred to as drought. Areas below the 100 - 300 mm/m^2 rainfall annually are accepted as arid lands and effects of drought are present on vegetation. Both salinity and drought cause osmotic stress on plants. Osmotic stress triggers early and late response mechanisms. Early response mostly includes involvement of stress perception molecules, signals, and transcription factors. Late response is triggered by stress signals after stress perception and utilizes antioxidant enzymes, molecular chaperons, LEA-like proteins, osmolyte biosynthesis. These responses serve three purposes as (1) maintaining physiological integrity, (2) regaining osmotic balance and (3) repairing damage caused by osmotic stress (Mahajan et al., 2005; Xiong and Zhu, 2002; Cramer et al., 2007).

In plant science, there are two basic approaches described as forward and reverse genetics which are utilized in different ways to understand molecular mechanism of stress tolerance. Forward genetics approach aims to identify genomic and proteomic differences underlying specific traits in selected mutant plants. In these studies, original varieties are present and comparison of original species against deliberately induced mutants or spontaneous mutations reveals proteomic and sequence changes behind the particular phenotype. Reverse genetics approach targets known sequence(s) and utilize silencing techniques to gain insight into the underlying function(s). As one of the widely used forward genetics approach, mutation breeding is a time and cost effective, robust, transferrable, ubiquitously applicable, non-hazardous, and environmentally friendly technique, which involves induction of mutations by physical (gamma rays, ion beam, neutrons, etc.) or chemical mutagens (ethyl methanesulfonate, EMS; sodium azide, Az; methylnitrosourea, MNU; etc.) and molecular characterization of obtained mutants (Sen and Alikamanoglu, 2014). In the last decade, 129 new mutant varieties belonging to 35 different cereal, fruit, legume, oil, fiber and tree plant species such as wheat, soybean, rice, tomato, sorghum, citrus, cotton, have been registered to Mutant Variety Database (MVD) of International Atomic Energy Agency (IAEE) from 28 different countries around the world. 90 of these mutants were developed by use of gamma rays, while 3 of these 90 mutants were soybean (IAEA 2020).

Advances in technology provide researchers with a wide range of techniques to characterize mutations in comparison to the original varieties. These techniques include direct sequencing, Li-Cor, High-

* Corresponding author

Performance Liquid Chromatography (HPLC), electrophoresis (in genomic, proteomic), capillary electrophoresis, high-resolution melt (HRM), matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF), next generation sequencing (NGS) technologies and more (Sikora et al., 2011; Bado et al., 2015). Among these, two-dimensional gel electrophoresis (2-DE) supported with MALDI-TOF is a strong and widely used technique due to its high-resolution results, which provide researchers data on protein structure, localization, relative concentration, post-translational modifications, functions, and interactions with other macromolecules. Crop breeding programs facilitate 2D proteomics to identify stress-responsive proteins by comparative proteomic analysis between control and the stressed plants. Developments in proteomic analysis techniques gave insight into the mechanisms of stress and stress response in plants. The conventional 2DE supported with mass spectrometry (MS) is extensively used in resolution of proteins and to detect the stress-induced alterations in the proteome composition of plants, recently (Zadražnik et al., 2013; Koh et al., 2015; Ahmad et al., 2017; Rahimi et al., 2019; Pandian et al., 2020; Frukh et al., 2020; Lu et al., 2020; Li et al., 2020).

In this study, proteomic profile alterations of S04-05 soybean variety and SM1 and SM3-1 mutants which originated from S04-05 variety were investigated under drought and salinity stresses. 54 proteins, which presented statistically significant alterations under both stress factors were characterized by MALDI-TOF. Identified protein spots were categorized by their functions and localization in plant cells.

Material and methods

Plant material and stress treatments for biochemical analysis

Biochemical responses of mutants (Atak et al., 2024) and S04-05 soybean variety to salinity and drought stresses were evaluated by total carbohydrate and proline contents. Drought and salinity tolerant SM1 and SM3-1 soybean mutants, which were generated in T.C. Istanbul Kultur University Plant Biotechnology Laboratory by mutation breeding, and their original variety S04-05 soybean variety were surface sterilized and germinated on Murashige and Skoog (MS) medium containing 30 g/L sucrose, 0.8% agar, 1 mg/L MS vitamin for biochemical analysis for 7 days. 90 mM NaCl and 10% PEG-6000 [Merck Polyethylene glycol 6000 for synthesis (8.07491.1000)] were added for the salinity and drought treatments, respectively. Seedlings were grown under 25 °C, 8/16 dark/light period for 14 days. Effects of salinity and drought were evaluated through growth parameters of average plant height and fresh weight. Thirty random plants of each group among a total of 180 plants were used for the growth evaluation.

Proline contents were measured according to Bates et al. (1973). Leaf tissue samples were extracted in 3% sulphosalicylic acid and filtered. Extracts were boiled at 100 °C for 1 h following the addition of acid ninhydrin reagent (ninhydrin and glacial acetic acid). Reaction was stopped by incubation in ice bath and fractions were separated by using toluene. Absorbance values were measured at 520 nm wavelength. Proline levels were calculated by using standard calibration curve in unit of μmol proline g^{-1} FW.

Effects of drought and salinity stress on total carbohydrate contents of soybean plants were evaluated by Dubois method (Dubois et al., 1956). 0.1 g of leaf tissues were grounded in distilled water and heated to 100 °C for 3 h. Samples were centrifuged at 10,000 g for 15 min. One mL of the supernatant was mixed with 1 mL of 80% (w/v) phenol and 5 mL of 96% sulfuric acid. The mixture was kept at room temperature for 10 min then incubated in 30 °C water for 30 min. The chromophore was measured at 490 nm. Total carbohydrate contents were calculated by using the standard calibration curve, which is prepared by 0.02, 0.04, 0.06, 0.08 mg/mL sucrose dilutions.

Effects of salinity stress on photosynthetic pigment contents of soybean plants were evaluated by Arnon method (Arnon, 1949). 0.1 g leaf tissue was grounded to fine powder by using liquid nitrogen. Pigments were extracted by adding 80% cold acetone. Extracts were filtered through Whatman No.2 filter paper. Samples were measured at 470, 645 and 663 nm, spectrophotometrically. Photosynthetic pigment contents were calculated according to the equations below:

(Chlorophyll a (Chll a) Concentration (mg/g· FW) =
11.24 × A663 – 2.04 × A645)

(Chlorophyll (Chll b) Concentration (mg/g· FW) =
20.13 × A645 – 4.19 × A663)

(Total Chlorophyll (mg/g· FW) =
7.05 × A663 + 13.09 × A645)

(Carotenoid (mg/g· FW) =
(1000 × A470 – 1.9 × Chll a – 63.14 × Chll b)/214)

Plant material and stress treatments for proteomic analysis

Drought and salinity tolerant SM1 and SM3-1 soybean mutants, which were generated in T.C. Istanbul Kultur University Plant Biotechnology Laboratory by mutation breeding, and their original variety S04-05 soybean variety were germinated in 9 × 5 well viols filled with perlite. 20 seeds of each variety were planted in three replicates. Plantlets were watered daily with Hoagland's nutrient solution for 14 days. Plantlets were grown in climate room conditions under 24 °C with 16 h light / 8 h dark period. After the 14th day, 90 mM NaCl was added to Hoagland's solution for salt stress treatment. Salt stress was applied for 7 days. For drought treatment, 14 days-old soybean plantlets watered with Hoagland's solution were subjected to total withheld watering for 5 days. Leaves of untreated, salt and drought stress treated plantlets were harvested and stored in -20 °C until further analysis.

Protein extractions

Total protein extractions were performed by trichloroacetic acid (TCA)/acetone method (Méchin et al., 2007). 0.3 g of leaf tissue was grounded to a fine powder in a pestle and mortar by liquid nitrogen. Samples were extracted by acetone containing 10% TCA and 1% 1,4-dithiothreitol (DTT). The samples were kept at -20 °C for 1 h and centrifuged at 25000 × *g* for 20 min. at 4 °C. The pellet was washed in acetone containing 1% DTT and incubated at -20 °C for 1 h to rinse the pigments from the samples. This step was repeated several times until observation of colorless sample pellets. The pellet was vacuum-dried and then dissolved in an 8 M urea buffer containing 20 mM DTT, 4% CHAPS, and 2% ampholyte (pH 3-10). The solution was vortexed extensively for 2 min. at room temperature and centrifuged twice at room temperature for 5 min. at 25000 × *g*. Then, the supernatant was collected. Samples were purified by using Bio-Rad ReadyPrep™ 2-D CleanUp Kit under manufacturer's instructions. Protein samples were quantified by the Lowry method (Lowry et al., 1951) with Nano Drop 1000 (Thermo Scientific) instrument in spectrophotometer mode.

Two-dimensional gel electrophoresis (2-DE)

17 cm Bio-Rad ReadyStrip™ IPG Strips (pH 3-10) (Bio-Rad #1632007) were rehydrated before protein loading. Active rehydration was conducted under 50 μA/IPG strip constant current in Bio-Rad PROTEAN IEF instrument. 1000 μg protein samples were diluted in 300 μL Bio-Rad 2-DE rehydration buffer (Bio-Rad, #1632106). Samples were separated on IPG strips submerged in focusing tray lines. Strips were covered with 2 - 3 mL of mineral oil to prevent loss of volume through vaporization. Isoelectric focusing (IEF) conditions were as follows: 1st step; 250 V for 20 min. in linear slope, 2nd step; 10000 V for 150 min. in linear slope, 3rd step; 10000 V for until 40000 voltage-hour was completed. A total of 50000 voltage-hour was applied approximately in 420 min.

After IEF, IPG strips were equilibrated in Bio-Rad equilibrium buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 2% DTT) and equilibrium buffer II (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) iodoacetamide) for 30 minutes. After the equilibrium process, IPG strips were placed on precast 22 × 24 cm 12% SDS PAGE gels. Gels were run in Dodeca Cell (Bio-Rad, USA) instrument under 16°C. Gel running was conducted under 35 mA for 30 min. and 48 mA for 5 h.

Gel staining and image analysis

Gels were incubated in fixation buffer containing 50% (v/v) ethanol and 2% phosphoric acid, overnight. Gels were rinsed three times with dH_2O for 30 min., then incubated in buffer containing 34% (v/v) methanol, 2% (v/v) phosphoric acid, 17% (w/v) ammonium sulfate for 1 h. Gels were stained in 0.05 (w/v) Coomassie brilliant blue added incubation buffer up-to 3 days under mild agitation. Protein spots were visualized in VersaDoc 4000MP system (Bio-Rad, USA) under white light. Images of stained gels are available as supplemental material.

After initial visualization, images were analyzed with PDQuest Advanced Software (Bio-Rad, USA). The quantity of each spot was normalized by using a local regression model. Based on average spot volume ratio, spots whose relative expression levels were altered (increase or decrease) in statistically significant manner among the compared groups were chosen for MALDI-TOF analysis. Statistical significance was assessed by using Student's *t*-test ($P < 0.01$). Protein regulations of the samples were compared according to the S04-05 control images. Spots that were up or down regulated more than 2-fold were considered regulated.

In-gel trypsin digestion and Zip-Tip C18 application

Proteins which presented statistically significant alterations were cut from gels by automated EXQuest Spot Cutter (Bio-Rad, USA). Selected protein spots were digested in 96-well plate by tryptic digestion method. Protein spots were destained on a rotary incubator in solution containing 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate. Proteins were reduced by breaking bisulfide bonds in 25 mM ammonium bicarbonate solution containing 10% Tris-(2-carboxyethyl)-phosphine (TCEP) at 60 °C for 10 min. For alkylation of samples, cystine side chains were methylated in 25 mM ammonium bicarbonate containing 100 mM 2-iodoacetamid (IAA) in room temperature for 1 h at dark. After the incubation, samples were transferred to 100% ACN. After rinsing ACN, samples were digested in 10 ng/µL trypsin overnight at 30 °C. Zip-Tip application of samples was started after equilibration of tips first in ACN and then in 0.1% trifluoroacetic acid. Each sample was eluted on MALDI plates by MALDI matrixes (4-α-cyanocinnamic acid) after intense mixing.

MALDI-TOF-TOF analysis

Protein identification experiments (MALDI-TOF MS and TOF/TOF tandem MS/MS) were performed by using ABSCIEX MALDI-TOF/TOF 5800 system (ABSCIEX, USA). The resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science Inc.) to identify proteins by ProteinPilot™ (ABSCIEX, USA) software (https://sciex.com) (Tortosa et al., 2017).

MALDI calibration was performed by internal and external standards. Each spectrum was gathered by an average of 200 laser hits. Only significant hits, as defined by the MASCOT probability analysis ($p < 0.05$) were accepted. The parameters for searching were enzyme of trypsin, 1 missed cleavage, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (M), peptide mass tolerance: 50 ppm, fragment mass tolerance: ±0.4 Da, peptide charge of 1^+ and monoisotopic.

Database analysis and pathway annotation of significantly altered proteins

Functional annotations of the identified proteins were performed by using a freely available classification system, PANTHER (http://www.pantherdb.org/). Classification of the identified proteins according to the biological processes and cellular localization were carried out by GO analysis using PANTHER annotation software. Protein-protein interaction network was constructed considering significantly altered proteins by STRING analysis software. Interaction analysis was carried out with an interaction score ≥ 0.9.

Statistical analysis

The experiments were repeated three times, and each data point was the mean of three replicates. Statistical evaluation of the means was performed by using one-way ANOVA analysis and statistically meaningful data were compared using Student-Newman-Keuls test performed by using GraphPad Prism version 8.0.1 for Windows statistics software (https://www.graphpad.com).

Results

Growth parameters

Effects of salinity and drought were evaluated through growth parameters of average plant height and weight. Both parameters decreased significantly in 90 mM NaCl and 10% PEG-6000 treated S04-05 variety (Fig. 1 and Fig. 3). Both SM1 and SM3-1 mutants which originated from S04-05 variety presented significantly increased values under both stress conditions compared to the treated S04-05 variety. Nevertheless, they both presented significant decreases compared to the untreated S04-05 variety (Fig. 2 and Fig. 4).

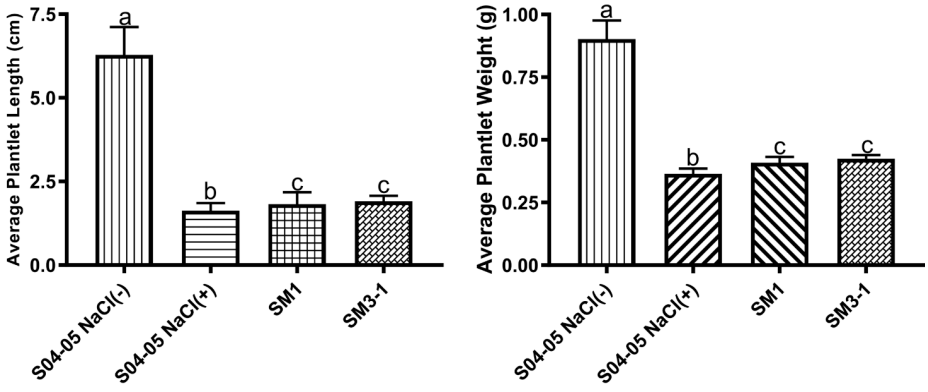

Fig. 1: Average plantlet lengths, and weights of untreated control and, 90 mM NaCl treated (14 days) S04-05 variety, SM1 and SM3-1 mutants. Different letters indicate statistically significant differences (P≤0.05).

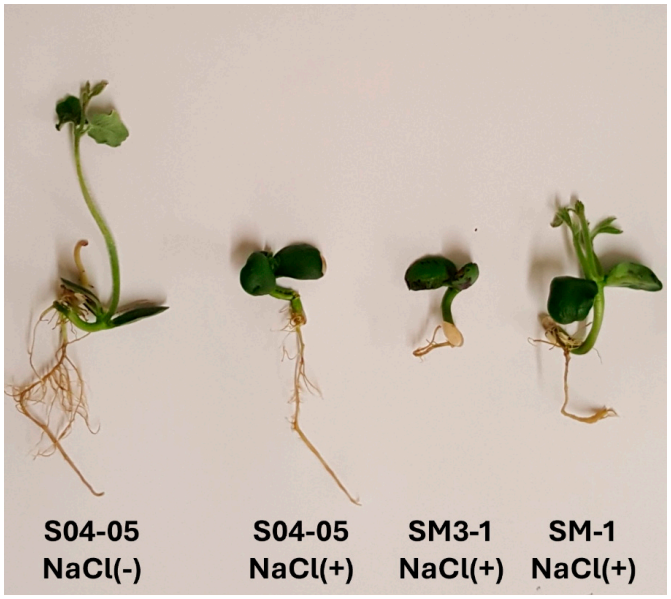

Fig. 2: Representation of untreated control and, 14 days 90 mM NaCl treated S04-05 variety, SM1 and SM3-1 mutant plantlets.

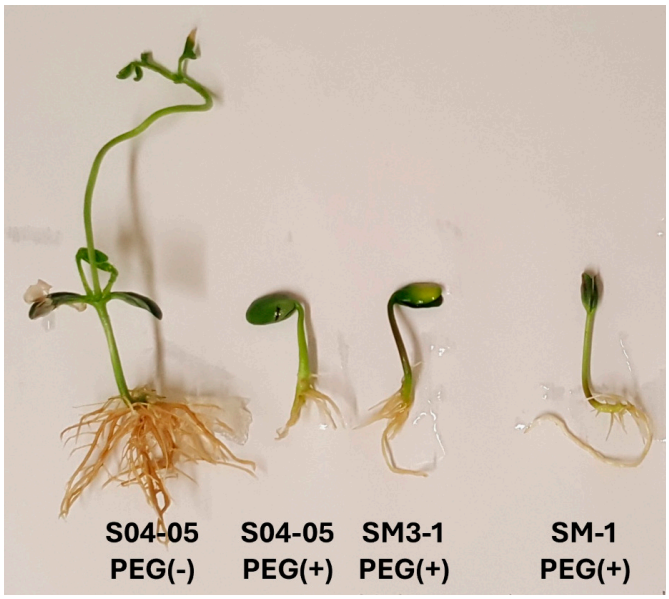

Fig. 4: Representation of untreated control and, 14 days %10 PEG treated S04-05 variety, SM1 and SM3-1 mutant plantlets.

Photosynthetic pigment contents

Total photosynthetic pigment contents of 90 mM NaCl and 10% PEG-6000 treated S04-05 variety presented statistically significant decreases (Fig. 5). Total chlorophyll content of S04-05 was decreased 61 and 58% following the PEG-6000 and NaCl treatments, respectively. Chlorophyll a decreased more than chlorophyll b compared to the untreated controls. Also, carotenoids were almost depleted in both stresses. Carotenoid content of S04-05 variety was decreased 93 and 90% following the PEG-6000 and NaCl treatments, respectively. In PEG-6000 treated SM1 mutant, total photosynthetic pigment content was not affected (8%) from the stress with slight difference in chlorophyll a / chlorophyll b ratio. In PEG-6000 treated SM3-1 mutant total chlorophyll content decreased in a statistically significant manner (23%), even though the values were significantly higher than PEG-6000 treated S04-05 variety. Additionally, carotenoid content of PEG-6000 treated SM3-1 mutant was significantly higher (11%) than both untreated and treated S04-05 variety. In 90 mM NaCl treated SM1 mutant, total chlorophyll levels increased significantly in expense of significantly decreased carotenoid levels. 90 mM NaCl treated SM3-1 mutant presented results similar to the PEG-6000 treated SM3-1 mutant as total chlorophyll content decreased (15%) in a statistically significant manner, even though the values were significantly higher than PEG-6000 treated S04-05 variety. Additionally, carotenoid content was significantly higher than both untreated and treated S04-05 variety.

Proline content

The proline content of S04-05 variety increased 1.54-fold after 90 mM NaCl treatment, while it decreased 0.47-fold after 10% PEG-6000 mediated drought treatment. Both SM1 and SM3-1 mutants which originated from S04-05 variety presented significantly increased proline contents (Fig. 6).

Total carbohydrate content

Total carbohydrate content of S04-05 variety decreased 0.59-fold after 90 mM NaCl treatment, while it increased 1.44-fold after 10% PEG-6000 mediated drought treatment. Both SM1 and SM3-1 mutants which originated from S04-05 variety presented significantly increased carbohydrate contents (Fig. 7).

Protein characterization

We evaluated protein profiles of soybean leaves from 21-day plantlets for salinity treatment and 19-day-old plantlets for drought treatment following the protein separation on 2-DE gels (Fig. S1 to

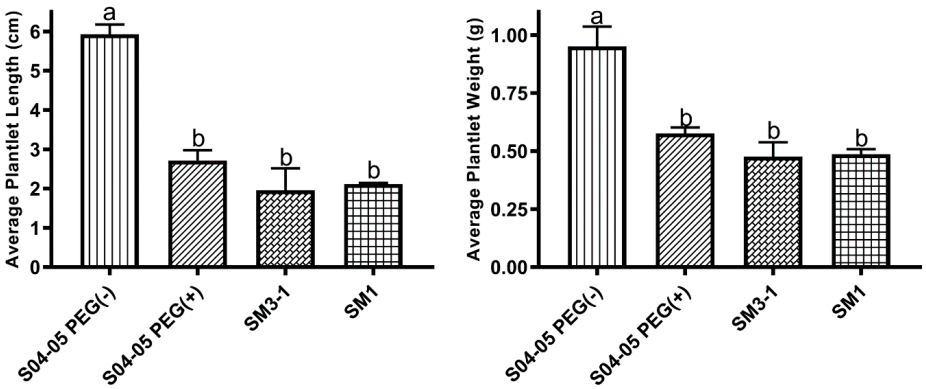

Fig. 3: Average plantlet lengths, and weights of untreated control and, 10% PEG treated (14 days) S04-05 variety, SM1 and SM3-1 mutants. Different letters indicate statistically significant differences (P≤0.05).

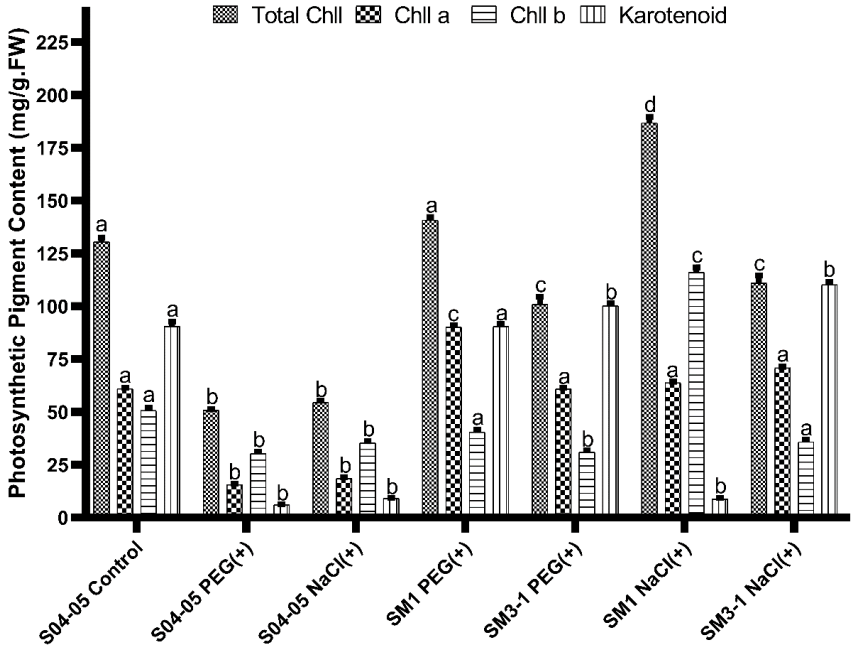

Fig. 5: Photosynthetic pigment content of untreated control and, 10% PEG treated (14 days) S04-05 variety, SM1 and SM3-1 mutants. Differences between average values represented by different letters are significant (P≤0.05).

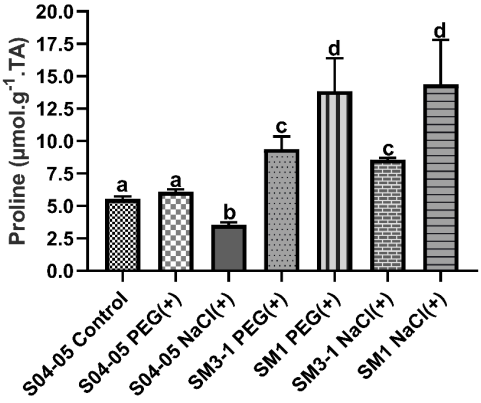

Fig. 6: Proline contents of salinity and drought treated S04-05 variety, SM1 and SM3-1 mutants. Differences between average values represented by different letters are significant (P≤0.05).

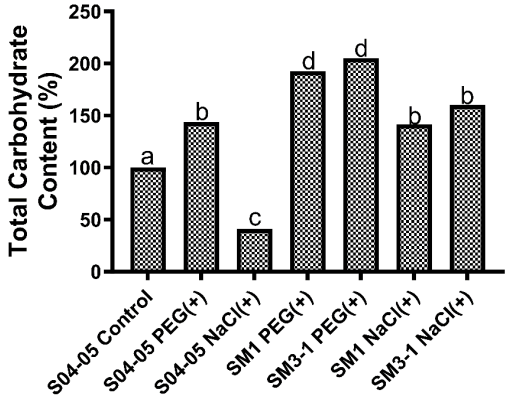

Fig. 7: Total carbohydrate contents of salinity and drought treated S04-05 variety, SM1 and SM3-1 mutants. Differences between average values represented by different letters are significant (P≤0.05).

Fig. S12). 54 protein spots presented over 2-fold regulation in all three biological replicates. These differentially regulated protein spots were excised and digested with trypsin in-gel and analyzed using an ABSCIEX MALDI-TOF/TOF 5800 system (Tab. 1 and 2). Three of these differentially regulated proteins were found to be salt induced proteins. These, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase, proteins were related to regulation of photosynthesis process. Also, among differentially regulated proteins putative fructose-bisphosphate aldolase 2 was found the most significant drought induced protein, which plays regulatory roles in photosynthesis and carbohydrate metabolism processes. UniProt protein database analysis of detected proteins for their localization in plant cells presented that 60% of proteins were chloroplastic. Also, 19% of proteins were localized in cytosol, 4% in mitochondria, 2% in nucleus, 2% in cell wall, 2% in cell membrane. 4% of proteins were found both in mitochondria and chloroplast, while the rest of 7% were localized in various other subcellular organelles (Fig. 8).

Proteins were also categorized by their functions in plant cells. Identified protein functions were carbohydrate metabolism (30%), photosynthesis (15%), antioxidant enzyme regulation (15%), amino acid metabolism (9%), ATP synthesis (9%), methylation (6%) and protein biosynthesis (4%). We also determined protein functions such as alkaloid biosynthesis, cell cycle control, cell wall peptidase activity, chlorophyll biosynthesis, transmembrane structure, and fatty acid metabolism by 2% for each (Fig. 9). Protein mass (kDa), protein score, UniProt Database protein names, localization, functions, and expression regulation of proteins (fold) were presented in Tab. 1 and 2.

Analysis of protein-protein interaction by STRING

Protein-protein interactions among the regulated proteins in response to salinity and drought were analyzed by the STRING analysis (Fig. 10).

Proteins belonging to various biological processes were involved. In the interaction network, three clusters were created according to their relations. Green colored protein cluster members were chloroplast or photosynthesis related proteins while red colored members were carbohydrate metabolism related. Blue colored members have roles in

Tab. 1: Differentially expressed proteins under salinity stress conditions.

Description	Protein Accession	Protein Mass (kDa)	Protein Score	Biological Process	Localization	Protein Regulation (Fold)		
						S04-05 Salinity (-)	SM1 Salinity (-)	SM3-1 Salinity (-)
Oxygen-evolving enhancer protein 2, chloroplastic [*Glycine max*]	Q38IX0	28212	72	Photosynthesis	Chloroplast	1	2.19	1.96
Phosphoribulokinase, chloroplastic [*Glycine max*]	I1J4L6	45269	376	Carbohydrate Metabolism	Chloroplast	1	1.01	1.06
ATP synthase CF1 alpha subunit (chloroplast) [*Glycine max*]	Q2PMS8	55719	590	ATP Synthesis	Chloroplast	1	1.03	1.29
ATP synthase subunit beta, mitochondrial [*Glycine max*]	I1NFS4	59809	626	ATP Synthesis	Mitochondria	1	1.11	0.73
Beta-amylase [*Glycine max*]	Q45UE7	56036	159	Carbohydrate Metabolism	Cytosol	1	0.16	0.16
Iron-superoxide dismutase [*Glycine max*]	Q71UA1	27498	342	Antioxidant Enzyme Regulation	Chloroplast	1	0.28	0.38
Superoxide dismutase [Fe], chloroplastic precursor [*Glycine max*]	P28759	27824	321	Antioxidant Enzyme Regulation	Chloroplast	1	0.41	0.64
Elongation factor Tu, chloroplastic-like [*Glycine max*]	Q43467	52175	435	Protein Biosynthesis	Chloroplast	1	0.41	1.12
ATP-dependent zinc metalloprotease FTSH, chloroplastic [*Glycine max*]	I1K7F0	74348	138	Photosynthesis	Chloroplast	1	3.15	13.51
Putative D-ribulose-5-phosphate-3-epimerase [*Glycine max*]	C6T4H4	29865	108	Photosynthesis	Chloroplast	1	0.51	1.64
Glutamine synthetase precursor [*Glycine max*]	Q95AG1	47624	408	Amino Acid Biosynthesis	Chloroplast	1	0.57	1.37
Glutamine synthetase leaf isozyme, chloroplastic [*Glycine max*]	I1MFC4	47662	342	Amino Acid Biosynthesis	Chloroplast	1	0.36	1.22
Transketolase, chloroplastic [*Glycine max*]	I1LFG4	80028	384	Carbohydrate Metabolism	Chloroplast	1	0.37	0.73
(R,S)-reticuline 7-O-methyltransferase [*Glycine max*]	G9I8U0	40893	256	Alkaloid Synthesis	Various	1	0.21	0.14
Carbonic anhydrase, chloroplastic isoform 3 [*Glycine max*]	K7KES9	29379	498	Carbohydrate Metabolism	Chloroplast	1	0.16	0.73
3‘-hydroxy-N-methyl-(S)-coclaurine 4‘-O-methyltransferase-like [*Glycine max*]	Q9LEL5	41482	341	Methylation	Various	1	0.76	0.09
Glyceraldehyde-3-phosphate dehydrogenase [*Glycine max*]	C6THM8	36818	359	Antioxidant Enzyme Regulation	Chloroplast	1	0.92	0.57

Description	Protein Accession	Protein Mass (kDa)	Protein Score	Biological Process	Localization	Protein Regulation (Fold)		
						S04-05 Salinity (+)	SM1 Salinity (+)	SM3-1 Salinity (+)
Phosphoribulokinase, chloroplastic [*Glycine max*]	I1J4L6	45269	338	Carbohydrate Metabolism	Chloroplast	1	1.62	1.48
ATP synthase CF1 alpha subunit (chloroplast) [*Glycine max*]	Q2PMS8	55719	751	ATP synthesis	Chloroplast	1	0.91	0.86
ATP synthase subunit beta, mitochondrial [*Glycine max*]	I1NFS4	59799	712	ATP synthesis	Mitocondria	1	1.06	0.92
RuBisCO large subunit-binding protein subunit beta, chloroplastic [*Glycine max*]	P08927	62805	631	Photosynthesis	Chloroplast	1	0.38	0.43
RuBisCO large subunit-binding protein subunit beta, chloroplastic [*Glycine max*]	P08927	62802	390	Photosynthesis	Chloroplast	1	0.58	0.46
20 kDa chaperonin, chloroplastic [*Glycine max*]	O65282	26653	180	Antioxidant Enzyme Regulation	Chloroplast/ Mitochondria	1	0.59	0.75
Adenosylhomocysteinase [*Glycine max*]	I1KS65	53251	319	Carbohydrate Metabolism	Cytosol	1	0.46	0.45
Enolase [*Glycine max*]	Q6RIB7	47628	123	Carbohydrate Metabolism	Cytosol	1	0.19	0.35
3‘-hydroxy-N-methyl-(S)-coclaurine 4‘-O-methyltransferase-like [*Glycine max*]	Q9LEL5	41482	896	Methylation	Various	1	0.54	0.3
Glutamate-1-semialdehyde 2,1-amino-mutase, chloroplastic [*Glycine max*]	I1JSD2	50011	190	Chlorophyll Biosynthesis	Chloroplast	1	0.14	0.23
Carbonic anhydrase, chloroplastic isoform 3 [*Glycine max*]	K7KES9	29379	362	Carbohydrate Metabolism	Chloroplast	1	3.75	3.07

Gamma-glutamyl hydrolase precursor [*Glycine max*]	P93164	37738	582	Cell Wall Peptidase Activity	Cell Wall	1	0.02	0.02
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [*Glycine max*]	Q38IX0	43175	550	Carbohydrate Metabolism	Chloroplast	1	2.06	1.5
Glyceraldehyde-3-phosphate dehydrogenase [*Glycine max*]	C6THM8	36818	624	Carbohydrate Metabolism	Chloroplast	1	0.44	0.36

Tab. 2: Differentially expressed proteins under drought stress conditions.

Description	Protein Accession	Protein Mass (kDa)	Protein Score	Biological Process	Localization	Protein Regulation (Fold)		
						S04-05 Drought (-)	SM1 Drought (-)	SM3-1 Drought (-)
Acyl-coenzyme A thioesterase PaaI domain-containing protein [*Glycine max*]	C6TGE4	17586	27	Fatty Acid Metabolism	Cytosol	1	1.55	0.96
Ribulose bisphosphate carboxylase/ oxygenase activase, chloroplastic-like [*Glycine max*]	D4N5G3	48635	568	Photosynthesis	Chloroplast	1	0.64	0.83
Oxygen-evolving enhancer protein 2, chloroplastic [*Glycine max*]	I1M712	28212	215	Photosynthesis	Chloroplast	1	1.4	0.41
Photosystem II stability/assembly factor HCF136, chloroplastic [*Glycine max*]	I1LPJ4	43142	436	Photosynthesis	Chloroplast	1	1.13	0.82
Uncharacterized protein LOC100803033 [*Glycine max*]	I1JV80	26640	343	Antioxidant Enzyme Regulation	Chloroplast / Mitocondria	1	1.38	0.72
ATP synthase CF1 alpha subunit (chloroplast) [*Glycine max*]	Q2PMS8	55719	573	ATP Synthesis	Chloroplast	1	1.35	1.11
Fructose-bisphosphate aldolase 1, chloroplastic [*Glycine max*]	I1JSJ3	42542	365	Carbohydrate Metabolism	Cytosol	1	1.3	0.86
Putative phosphoglycerate kinase [*Glycine max*]	K7LXU3	50036	624	Carbohydrate Metabolism	Cytosol	1	0.65	1.09
Glutamine synthetase precursor [*Glycine max*]	Q95AG1	47624	457	Amino Acid Biosynthesis	Chloroplast	1	1.09	0.82
Ferredoxin--NADP reductase, leaf isozyme, chloroplastic [*Glycine max*]	Q9FKW6	40545	342	Antioxidant Enzyme Regulation	Chloroplast	1	0.23	0.06
Triosephosphate isomerase isoform X1 [*Glycine max*]	Q6GW08	27187	425	Carbohydrate Metabolism	Cytosol	1	4.21	0.88
Putative fructose-bisphosphate aldolase 2 [*Glycine max*]	C6TJM1	42843	510	Carbohydrate Metabolism	Cytosol	1	2.16	0.87
Fructose-bisphosphate aldolase 1, chloroplastic [*Glycine max*]	I1JSJ3	42925	226	Carbohydrate Metabolism	Chloroplast	1	4.16	3.6
Uncharacterized protein LOC100813910 [*Glycine max*]	K7K7H0	39822	104	Cell Cycle Regulation	Nucleus	1	0.01	3.03

Description	Protein Accession	Protein Mass (kDa)	Protein Score	Biological Process	Localization	Protein Regulation (Fold)		
						S04-05 Drought (+)	SM1 Drought (+)	SM3-1 Drought (+)
Elongation factor Tu, chloroplastic-like [*Glycine max*]	Q43467	52175	315	Protein Biosynthesis	Chloroplast	1	0.45	0.5
Glutamine synthetase precursor [*Glycine max*]	Q95AG1	47624	289	Amino Acid Biosynthesis	Chloroplast	1	0.75	0.63
Putative phosphoglycerate kinase [*Glycine max*]	K7LXU3	50036	744	Carbohydrate Metabolism	Cytosol	1	0.79	0.98
Glutamine synthetase leaf isozyme, chloroplastic [*Glycine max*]	I1MFC4	47662	255	Amino Acid Biosynthesis	Chloroplast	1	0.62	0.38
Putative fructose-bisphosphate aldolase 2 [*Glycine max*]	C6TJM1	42843	188	Carbohydrate Metabolism	Cytosol	1	1.04	1.09
LRR receptor-like serine/threonine-protein kinase GSO2 [*Glycine max*]	K7LGZ7	44507	24	Transmembrane Protein	Membrane	1	1.86	0.03
3‘-hydroxy-N-methyl-(S)-coclaurine 4‘-O-methyltransferase-like [*Glycine max*]	Q9LEL5	41482	196	Methylation	Various	1	0.02	0.09
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [*Glycine max*]	Q38IX0	43175	407	Carbohydrate Metabolism	Chloroplast	1	0.6	0.6

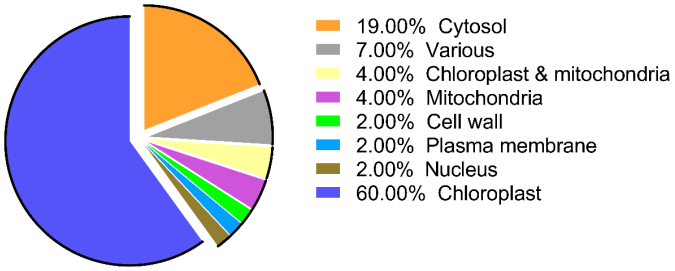

Fig. 8: Cellular localizations of characterized proteins.

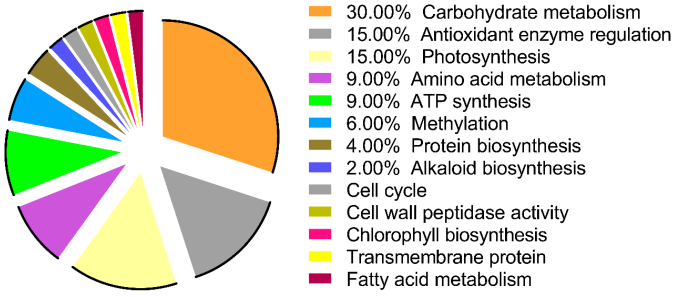

Fig. 9: Biological processes of characterized proteins.

various other biological processes as listed previously. KEGG pathways of significantly altered proteins were also generated for photosynthesis (Fig. 11) and pentose phosphate pathways (Fig. 12).

Discussion

Plants are constantly exposed to one or more biotic and abiotic stresses including salinity and drought due to the accelerating effects of global warming. Salinity causes Na^+ toxicity and ionic imbalance in plant cells, therefore, leads to deleterious metabolic alterations through protein synthesis, enzymatic reactions, and more. Excess Na^+ competes with other essential macro and micronutrients (Frukh et al., 2020). Besides, salinity reduces CO_2 fixation through crippling stomatal conductance by its osmotic effects, which ultimately impair the rate of photosynthesis. Plant physiological processes are also directly or indirectly influenced by water scarcity. Drought impairs the rate of photosynthesis through reducing stomatal conductance as well as altering other morphological and physiological traits as leaf water potential and sap movement (Çelik et al., 2017). Many studies have been conducted to evaluate the responses of plants to salinity and drought stresses and demonstrated that both stresses lower plant growth significantly, if the varieties are not tolerant or resistant. Depending on plant species and varieties, salinity is reported to have a greater impact than drought stress or vice versa. We also evaluated the effects of both stresses on plant growth through average plant weight and height parameters and determined the detrimental effects

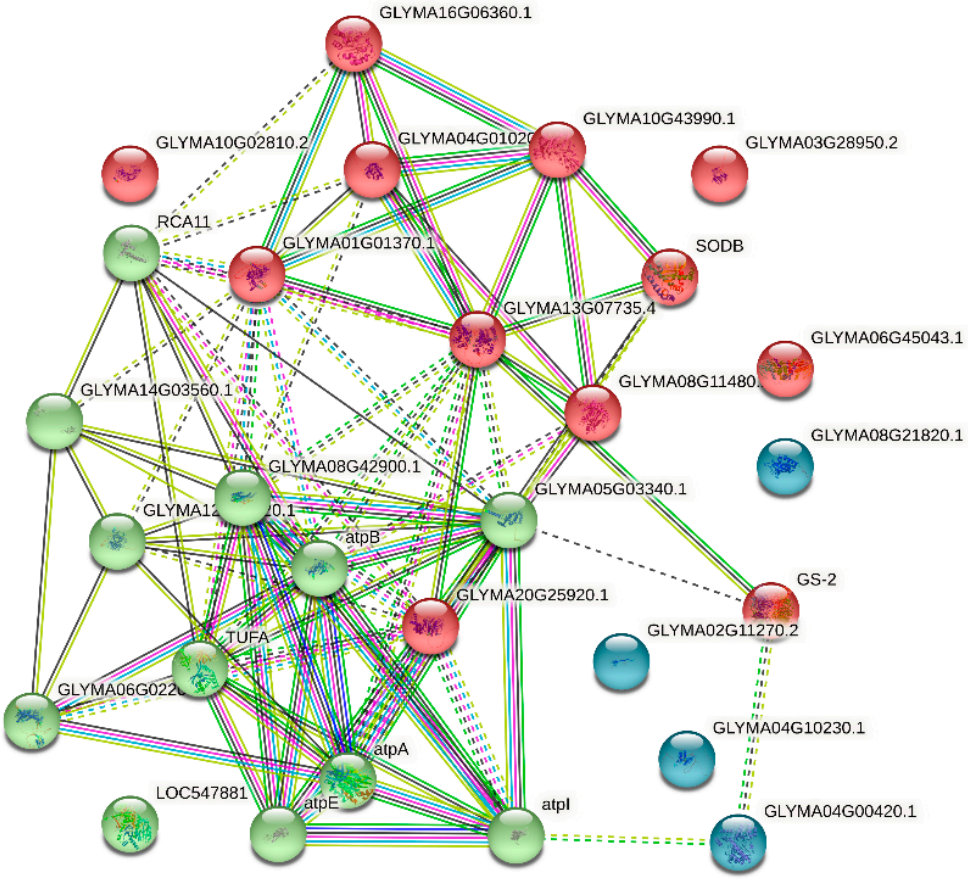

Fig. 10: Protein-protein interaction network constructed using STRING analysis software. Light blue line: from curated database, pink line: experimentally determined, red line: gene fusion, dark blue line: gen co-occurrence, purple line: co-expression. GLYMA_04G01020: Fructose-bisphosphate aldolase; GLYMA_01G01370.1: Phosphoribulokinase; GLYMA_13G07735.4: Phosphoglycerate kinase; SODB: Superoxide dismutase [Fe], chloroplastic; GS-2: Gln synthetase, chloroplastic; GLYMA04G00420.1: Class-III pyridoxal-phosphate-dependent aminotransferase family; atpB: ATP synthase subunit beta, chloroplastic; TUFA: Elongation factor Tu, chloroplastic; atpA: ATP synthase subunit alpha, mitochondrial; atpE: ATP synthase epsilon chain.

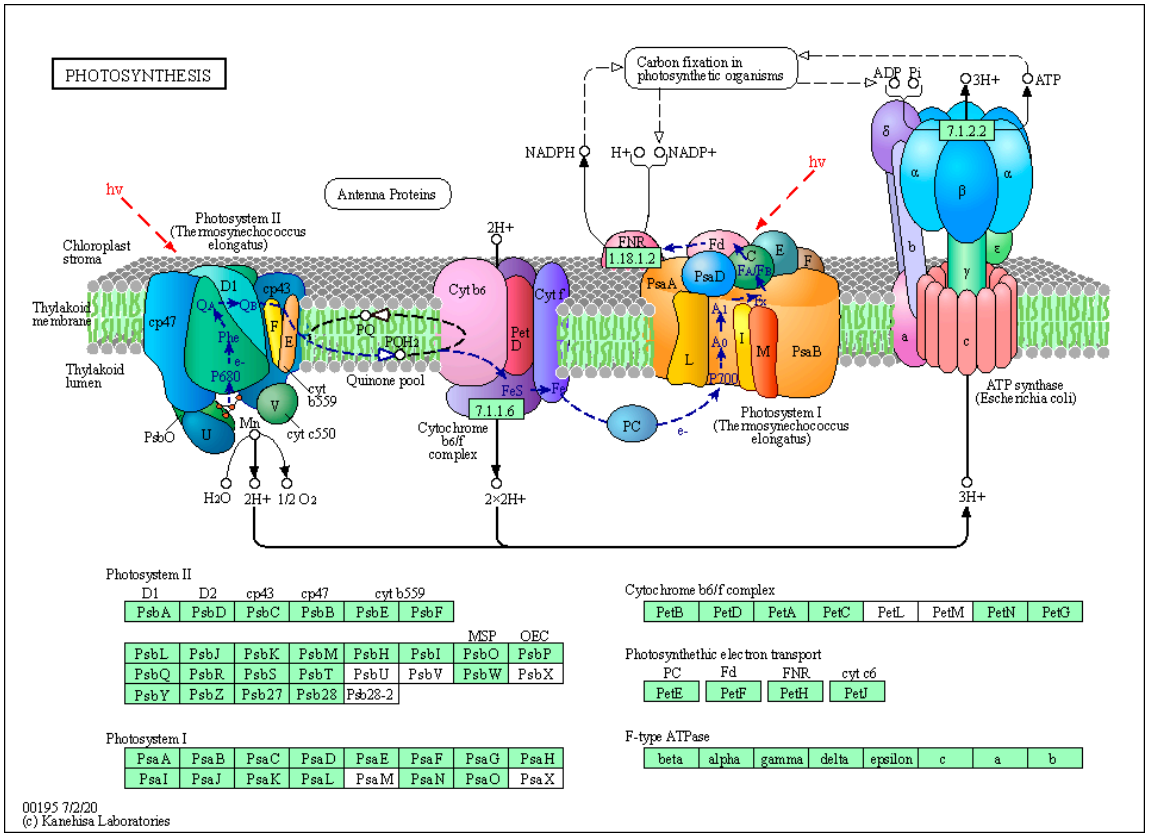

Fig. 11: Photosynthesis scheme constructed from the significantly altered proteins in KEGG pathway analysis. Protein accessions highlighted as green are the hotspots which were altered by gamma radiation in favor of salinity and drought tolerance.

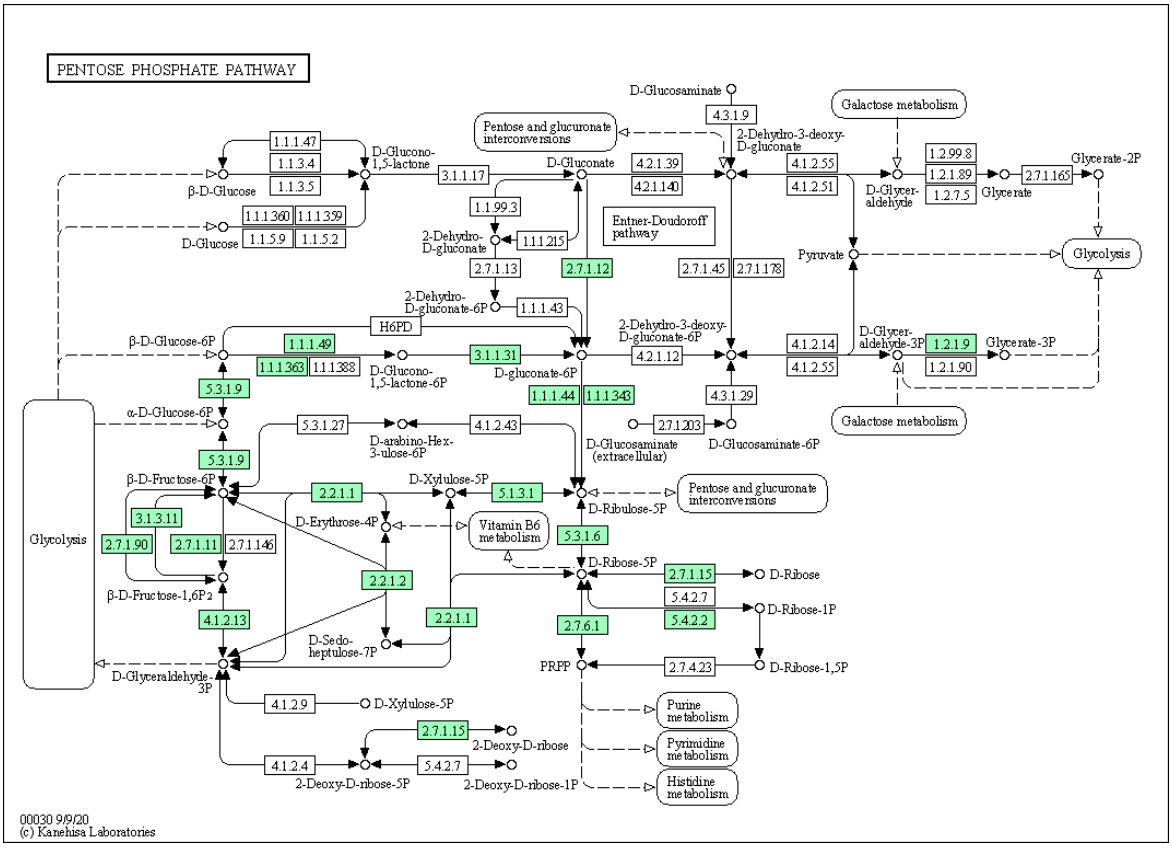

Fig. 12: Pentose phosphate pathway constructed from the significantly altered proteins in KEGG pathway analysis. Protein accessions highlighted as green are the hotspots which were altered by gamma radiation in favor of salinity and drought tolerance.

in accordance with previous publications. However, photosynthetic pigment contents of SM1 and SM3-1 were not negatively affected since both gamma-induced mutants are salinity and drought tolerant. Therefore, we evaluated proline levels as one of the key osmoprotectants which may contribute to the protection of photosynthetic machinery to this extent.

Proline is a multifunctional amino acid, which contributes to osmotic tolerance as well as various physiological processes in plants. It has central roles in oxidative pentose phosphate metabolism by supplying $NAD(P)^+$ in cytosol. In pentose phosphate pathway, $NAD(P)^+$ is reduced to $NAD(P)H^+$ and generates carbon dioxide. Therefore, under osmotic stress conditions such as salinity and drought, continuity of carbon reduction can be maintained. On the other hand, NAD(P)H is used in proline biosynthesis to prevent ROS production. Proline also regulates osmotic homeostasis adjustment and helps plants to restore water content particularly under osmotic stresses (El Moukhtari et al., 2020). In this context, we evaluated the proline contents of S04-05 variety and its mutants to interpret their combined salinity and drought tolerances. Significantly increased proline contents in both of our mutants under salinity and drought stresses, support the general scientific knowledge on the osmotic adjustment role of proline for drought and salinity tolerance in soybean and many other plants (Çelik et al., 2017). Considering involvement of proline metabolism on pentose phosphate pathway, we also investigated effects of both osmotic stresses on total carbohydrate contents. Carbohydrates as starch act as key molecules in plant responses to various abiotic stresses. Under growth limiting conditions, plants utilize these carbohydrates in alternative processes to provide energy and carbon for crucial metabolic pathways. These carbohydrates may act as osmoprotectants and may also support other compatible solutes to mitigate the detrimental effects of stress. Moreover, in ABA-dependent signaling, carbohydrates act as signal molecules and crosstalk with the components of the pathway to activate downstream components in the stress response (Thalmann and Santelia, 2017). Hence, significantly increased total carbohydrate contents in both our salinity and drought tolerant mutants suggest involvement of these molecules in enhancing both stress tolerances, comparing to their untreated and stressed original variety (S04-05).

2-DE is a powerful tool to understand plant growth and development processes. 2-DE, which may give insight on proteomic characterization of different plants, identification of proteins, measurement of nutritional value of a plant, understanding of stress response, identification of important molecular markers, is widely used in biotechnological plant breeding. In this study, we investigated 2-D proteomic profile alterations of two soybean mutants (SM1 and SM3-1), which can tolerate salinity and drought in higher intensities compared to their original variety.

We identified increased basal levels of chloroplastic oxygen-evolving enhancer protein, phosphoribulokinase, ATP-dependent zinc metalloprotease, ATP synthase CF1 alpha subunit in both untreated mutants comparing to the original S04-05 variety, while elongation factor Tu, D-ribulose-5-phosphate-3 epimerase, glutamine synthetase precursor and chloroplastic glutamine synthetase leaf isozyme increased only in untreated SM3-1 mutant. Mitochondrial ATP synthase subunit beta was the only protein increased just in SM1 mutant.

Moreover, we identified three proteins which were found related to salinity tolerance in previous studies. Glyceraldehide-3-phosphate dehydrogenase (GAPDH), which was one of the significantly increased salinity-induced proteins, is related to salinity, ABA and osmotic stress tolerances. Zhang et al. (2011) obtained *OsGAPDH3* overexpressing transgenic rice plants and reported increased salt tolerance. Moreover, GAPDH was related to regulation of hydrogen peroxide levels and reducing salinity toxicity (Zhang et al., 2011). GAPDH catalyzes the conversion of glyceraldehide-3-phosphate to 1,3-biphosphoglycerate. This process converts NAD^+ to high energy electron carrier NADHs (Martin and Cerff, 1986). $NADP^+$ specific GAPDH takes part in the fixation of photosynthetic CO_2 in chloroplasts (Cerff and Chambers, 1979). Salt tolerant SM1 and SM3-1 mutants presented 2.06- and 1.5-fold increased glyceraldehyde-3-phosphate dehydrogenase A profiles compared to the original S04-05 variety, respectively. RUBISCO has roles in carboxylation of phosphoribulokinase and sedoheptulose biphosphatease proteins. It is also part of the reduction processes of Calvin cycle. It catalysis carbon fixation in higher plants. In their 2D proteomic study, Ngara et al. (2012) reported that RUBISCO levels in salt treated sorghum plants were elevated in response to increased energy and photosynthesis requirements of plants. In accordance with this increase, they also reported a decrease in other proteins of the mechanism such as phosphoribulokinase and sedoheptulose biphosphatease. They suggested that different processes of Calvin cycle may be regulated alternatively due to the effects of salt stress (Ngara et al., 2012). In our study, SM1 and SM3 mutants presented 0.38- and 0.43-fold decreased RUBISCO expression with respect to stressed control plants. However, phosphoribulokinase, which converts ribulose-5-kinase to ribulose-1-5-biphosphate increased 1.62- and 1.48-fold. During various stresses, exacerbated photosynthetic activity is reported in sensitive cultivars. Especially carbon fixation enzymes as fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase are typically reduced (Michaletti et al., 2018). In both of our salinity tolerant mutants, glyceraldehyde-3-phosphate dehydrogenase A and phosphoribulokinase were enhanced. Moreover, carbonic anhydrase, which catalysis the formation of carbonic acid by reacting water and carbon dioxide molecules, was increased by 3.75- and 3.07-fold in SM3-1 and SM1 mutants under salt stress, respectively. We suggest that elevated phosphoribulokinase, GAPDH and carbonic anhydrase expressions may have compensated for the decreased RUBISCO levels and contributed to the salt tolerance capacity of our soybean mutants.

In untreated mutants, ATP synthase CF1 alpha subunit and fructose-biphosphate aldolase 1 basal levels were increased comparing to the original S04-05 variety. Oxygen-evolving enhancer protein, photosystem II stability/assembly factor HCF136, fructose biphosphate aldolase 1, thioesterase Paal domain-containing protein, triosephosphate isomerase isoform X1, fructose biphosphate aldolase 2 and acyl-coenzyme A thioesterase PaaI domain-containing protein basal levels were only increased in SM3-1 mutant. Phosphoglycerate kinase was the only protein, which was increased only in SM-1 mutant. Also, 9 of the 54 identified protein spots which were aligned between 3 and 10 pH-values were regulated after drought treatment. These proteins were elongation factor Tu, glutathione synthetase, phosphoglycerate kinase, glutamine synthetase leaf isozyme, fructose biphosphate aldolase 2, LRR receptor like serine/threonine protein kinase,3'-hidroxy-N-methyl-(S)codaurin4'-0-methyltransferase,glyseraldehide-3- phosphate dehydrogenase A. Among these proteins, fructose biphosphate aldolase 2 presented significant increase in both mutants. LRR receptors like serine/threonine protein kinase is the only significantly increased protein in SM1 mutant as 1.86-fold. Other proteins presented decreases in both mutants compared to their original variety S04-05. Castillejo et al. (2008) analyzed 2D proteomic profiles of sunflower (*Helianthus annuus*) plants under drought stress and found out that phosphoglycerate kinase, GAPDH and fructose biphosphate aldolase protein intensities were decreased significantly (Castillejo et al., 2008). Çevik et al. (2019) identified 24, differentially regulated proteins in a comparative 2-DE-DIGE study between drought tolerant and sensitive chickpea (*Cicer arietinum*) plants. Photosynthesis and energy metabolism related protein expressions were increased significantly in the drought-tolerant variety while decreased in the sensitive variety. Moreover, cytosolic fructose biphosphate aldolase protein expression was among the most significant drought induced proteins as in our results (Çevik et al.,

2019). Fan et al. (2009) also reported that fructose-1,6-biphosphate aldolase was strongly expressed in both drought and salinity stress treated *Sesuvium portulacastrum* plants. After transformation studies, they expressed *Sesuvium portulacastrum* fructose-1,6-biphosphate aldolase protein in *E. coli* and obtained salinity resistance as well. These results suggested involvement of this protein in survival of *Sesuvium portulacastrum* under intense drought and salinity conditions (Fan et al., 2009). Khueychai et al. (2015) reported increased fructose-1,6-biphosphate aldolase expression in both leaf and root tissues of drought treated sugarcane plants (Khueychai et al., 2015). GAPDH protein expression decreased in the drought tolerant fennel plants compared to the susceptible varieties (Khodadadi et al., 2017). Likewise, in our study, GAPDH protein expression was reduced in both of our mutants under drought stress. LRR-RLK protein expression was specifically suggested to be involved in rapid response to drought stress in tolerant plant varieties (Marshall et al., 2012). In our SM1 soybean mutant LRR-RLK protein was found 1.86-fold increased under drought stress conditions. Yu et al. (2019) also reported LRR-RLK protein as one of the related QTL regions containing candidate genes in root development and water uptake functions during drought stress (Yu et al., 2019). 2D proteomic studies also revealed that in sunflower plants, which were subjected to drought stress by using PEG 6000 increases caffeoyl-CoA 3-O-methyltransferase expression (Fulda et al., 2011).

Conclusion

Salinity and drought stress are two main global issues in many agricultural plants as the result of global warming. Both stresses affect plant growth closely as well as productivity and yield. Two-dimensional electrophoresis proteomic profiling of plants under abiotic stress factors is crucial for several reasons. 2-DE allows researchers to understand plant stress responses by observing global changes in the plant proteome, providing insights into which proteins are upregulated or downregulated in response to stress. 2-DE enables the identification of stress-responsive proteins by the separation of proteins based on isoelectric point and molecular weight, helping to identify stress-responsive proteins, including enzymes, regulatory proteins, and structural proteins. These proteins can provide clues about the mechanisms that plants use to cope with stress, such as oxidative stress management, ion transport, or osmotic adjustment. Abiotic stress often induces post-translational modifications (PTMs) that alter protein activity, stability, or localization. 2-DE combined with staining methods or mass spectrometry can also detect PTMs, revealing critical regulatory mechanisms. 2-DE also provides a snapshot of the proteome under specific stress conditions, offering a systemic view of the metabolic and signaling pathways activated in plants. It complements other omics approaches for a comprehensive understanding of stress responses. Understanding how plants respond to abiotic stresses is increasingly important in the context of climate change, where extreme weather conditions are becoming more frequent. 2-DE profiling can help identify traits and proteins critical for adapting to changing environmental conditions. Knowledge gained from 2-DE studies can inform agronomic practices, such as the application of protective agents or the use of bioengineering techniques to enhance plant resilience. Therefore, identifying both salinity and drought related proteins in soybean leaves spotlighted biological processes variations of our gamma radiation induced mutants. Especially, photosynthesis and carbohydrate metabolisms altered significantly in favor of salinity and drought tolerances combined.

Acknowledgement

This study was financially supported by the Republic of Türkiye Ministry of Agriculture and Forestry General Directorate of Agricultural Research and Policies (TAGEM) (Grant Number: TAGEM-17-AR-GE-56).

Conflict of interest

No potential conflict of interest was reported by the authors.

References

Ahmad, J., Bashir, H., Bagheri, R., et al., 2017: Drought and salinity induced changes in ecophysiology and proteomic profile of *Parthenium hysterophorus*. PLoS One. 12(9):e0185118. DOI: 10.1371/journal.pone.0185118

Bado, S., Forster, B.P., Nielen, S., et al., 2015: Plant Mutation Breeding: Current Progress and Future Assessment. In: Plant Breeding Reviews: Volume 39. John Wiley & Sons, Inc., 23-88. DOI: 10.1002/9781119107743.ch02

Bates, L.S., Waldren, R.P., Teare, I.D., 1973: Rapid determination of free proline for water-stress studies. Plant Soil. 39, 205-207. DOI: 10.1007/BF00018060

Bellaloui, N., 2012: Soybean seed phenol, lignin, and isoflavones and sugars composition altered by foliar boron application in soybean under water stress. Food Nutr. Sci. 3, 579. DOI: 10.4236/fns.2012.34080

Castillejo, M.A., Maldonado, A.M., Ogueta, S., Jorrin, J.V., 2008: Proteomic analysis of responses to drought stress in sunflower (*Helianthus annuus*) leaves by 2DE gel electrophoresis and mass spectrometry. Open Prot. J. 1, 59-71. DOI: 10.2174/1875039700801010059

Çelik, Ö., Ayan, A., Atak, Ç., 2017: Enzymatic and non-enzymatic comparison of two different industrial tomato (*Solanum lycopersicum*) varieties against drought stress. Bot. Stud. 58, 1-13. DOI: 10.1186/s40529-017-0186-6

Cerff, R., Chambers, S.E., 1979: Subunit structure of higher plant glyceraldehyde-3-phosphate dehydrogenases (EC 1.2.1.12 and EC 1.2.1.13). DOI: 10.1016/S0021-9258(18)50523-8

Çevik, S., Akpinar, G., Yildizli, A., et al., 2019: Comparative physiological and leaf proteome analysis between drought-tolerant chickpea *Cicer reticulatum* and drought-sensitive chickpea *C. arietinum*. J. Biosci. 44, 1-13. DOI: 10.1007/s12038-018-9836-4

Cramer, G.R., Ergül, A., Grimplet, J., et al., 2007: Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. Funct. Integr. Genomics. 7, 111-134. DOI: 10.1007/s10142-006-0039-y

Dubois, M., Gilles, K.A., Hamilton, J.K., et al., 1956: Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356. DOI: 10.1021/ac60111a017

El Moukhtari, A., Cabassa-Hourton, C., Farissi, M., Savouré, A., 2020: How does proline treatment promote salt stress tolerance during crop plant development? Front Plant Sci. 11, 1127. DOI: 10.3389/FPLS.2020.01127/BIBTEX

Fan, W., Zhang, Z., Zhang, Y., 2009: Cloning and molecular characterization of fructose-1,6-bisphosphate aldolase gene regulated by high-salinity and drought in *Sesuvium portulacastrum*. Plant Cell Rep. 28, 975-984. DOI: 10.1007/s00299-009-0702-6

FAO, 2020: FAOSTAT. Retrieved 26th August 2020, from http://www.fao.org/faostat/en/#data.

Frukh, A., Siddiqi, T.O., Khan, M.I.R., Ahmad, A., 2020: Modulation in growth, biochemical attributes, and proteome profile of rice cultivars under salt stress. Plant Physiol. Biochem. 146, 55-70. DOI: 10.1016/j.plaphy.2019.11.011

Fulda, S., Mikkat, S., Stegmann, H., Horn, R., 2011: Physiology and proteomics of drought stress acclimation in sunflower (*Helianthus annuus* L.). Plant Biol. 13, 632-642. DOI: 10.1111/j.1438-8677.2010.00426.x

Hirt, H., Shinozaki, K., 2004: Plant responses to abiotic stress. Springer Science & Business Media.

IAEA, 2020: Mutant Variety Database (MVD). Retrieved 28th August 2020 from https://mvd.iaea.org/.

KHODADADI, E., FAKHERI, B.A., AHARIZAD, S., et al., 2017: Leaf proteomics of drought-sensitive and -tolerant genotypes of fennel. Biochim. Biophys. Acta - Proteins Proteomics 1865, 1433-1444. DOI: 10.1016/j.bbapap.2017.08.012

KHUEYCHAI, S., JANGPROMMA, N., DADUANG, S., et al., 2015: Comparative proteomic analysis of leaves, leaf sheaths, and roots of drought-contrasting sugarcane cultivars in response to drought stress. Acta Physiol. Plant. 37, 88. DOI: 10.1007/s11738-015-1826-7

KOH, J., CHEN, G., YOO, M.J., et al., 2015: Comparative proteomic analysis of *Brassica napus* in response to drought stress. J. Proteome Res. 14, 3068-3081. DOI: 10.1021/pr501323d

LI, G.-Z., LI, H.-X., XU, M.-J., et al., 2020: Functional characterization and regulatory mechanism of wheat CPK34 kinase in response to drought stress. BMC Genomics. 21, 577. DOI: 10.1186/s12864-020-06985-1

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J., 1951: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275. DOI: 10.1016/S0021-9258(19)52451-6

Lu, L.-M., Yang, S.-Y., Liu, L., et al., 2020: Physiological and quantitative proteomic analysis of NtPRX63-overexpressing tobacco plants revealed that NtPRX63 functions in plant salt resistance. Plant Physiol. Biochem. 154, 30-42. DOI: 10.1016/j.plaphy.2020.04.022

MAHAJAN, S., TUTEJA, N., 2005: Cold, salinity and drought stresses: an overview. Arch. Biochem. Biophys. 444(2), 139-158. DOI: 10.1016/j.abb.2005.10.018

MARSHALL, A., AALEN, R.B., AUDENAERT, D., et al., 2012: Tackling drought stress: RECEPTOR-LIKE KINASES present new approaches. Plant Cell. 24, 2262-2278. DOI: 10.1105/tpc.112.096677

MARTIN, W., CERFF, R., 1986: Prokaryotic features of a nucleus-encoded enzyme: cDNA sequences for chloroplast and cytosolic glyceraldehyde-3-phosphate dehydrogenases from mustard (*Sinapis alba*). Eur. J. Biochem. 159, 323-331. DOI: 10.1111/j.1432-1033.1986.tb09871.x

MÉCHIN, V., DAMERVAL, C., ZIVY, M., 2007: Total protein extraction with TCA-acetone. In: Plant Proteomics, 1-8. Springer. DOI: 10.1385/1-59745-227-0:1

MICHALETTI, A., NAGHAVI, M.R., TOORCHI, M., ZOLLA, L., RINALDUCCI, S., 2018: Metabolomics and proteomics reveal drought-stress responses of leaf tissues from spring-wheat. Scientific reports 9; 8(1), 1-8. DOI: 10.1038/s41598-018-24012-y

NGARA, R., NDIMBA, R., BORCH-JENSEN, J., et al., 2012: Identification and profiling of salinity stress-responsive proteins in *Sorghum bicolor* seedlings. J. Proteomics 75, 4139-4150. DOI: 10.1016/j.jprot.2012.05.038

PANDIAN, S., RAKKAMMAL, K., SAGINA, R.A., et al., 2020: Abiotic stress and applications of omics approaches to develop stress tolerance in agronomic crops. In: Agronomic Crops, 557-578. Springer Singapore. DOI: 10.1007/978-981-15-0025-1_26

PARIDA, A.K., DAS, A.B., 2005: Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60, 324-349. DOI: 10.1016/j.ecoenv.2004.06.010

RAHIMI, M., KORDROSTAMI, M., MORTEZAVI, M., S.C.S., 2019: Identification of drought-responsive proteins of sensitive and tolerant tea (*Camellia sinensis* L.) clones under normal and drought stress conditions. Curr. Proteomics 17, 227-240. DOI: 10.2174/1570164617666191016094142

RAO, K.M., RAGHAVENDRA, A.S., REDDY, K.J., 2006: Physiology and molecular biology of stress tolerance in plants. Springer Science & Business Media.

SEN, A., ALIKAMANOGLU, S., 2014: Characterization of drought-tolerant sugar beet mutants induced with gamma radiation using biochemical analysis and isozyme variations. J. Sci. Food Agric. 94(2), 367-72. DOI: 10.1002/jsfa.6393

THALMANN, M., SANTELIA, D., 2017: Starch as a determinant of plant fitness under abiotic stress. New Phytol. 214, 943-951. DOI: 10.1111/NPH.14491

TORTOSA, M., CARTEA, M.E., RODRÍGUEZ, V.M., VELASCO, P., 2017: 'Omic' profiling of *B. oleracea* challenged with *Xanthomonas campestris* pv. *campestris*. In: VII International Symposium on Brassicas 1202, 63-68. DOI: 10.17660/ActaHortic.2018.1202.9

XIONG, L., ZHU, J., 2002: Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ. 25, 131-139. DOI: 10.1046/j.1365-3040.2002.00782.x

YU, X., BROWN, J.M., GRAHAM, S.E., et al., 2019: Detection of quantitative trait loci associated with drought tolerance in St. Augustinegrass. PLoS One. 14(10):p.e0224620. DOI: 10.1371/journal.pone.0224620

ZADRAŽNIK, T., HOLLUNG, K., EGGE-JACOBSEN, W., et al., 2013: Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.). J. Proteomics 78, 254-272. DOI: 10.1016/j.jprot.2012.09.021

ZHANG, X.H., RAO, X.L., SHI, H.T., et al., 2011: Overexpression of a cytosolic glyceraldehyde-3-phosphate dehydrogenase gene OsGAPC3 confers salt tolerance in rice. Plant Cell Tissue Organ Cult. 107, 1-11. DOI: 10.1007/s11240-011-9950-6

ORCID
Alp Ayan https://orcid.org/0000-0003-3749-0472
Sinan Meriç https://orcid.org/0000-0003-0336-0004
Tamer Gümüş https://orcid.org/0000-0002-5674-4987
Özge Çelik https://orcid.org/0000-0002-9565-5135
Çimen Atak https://orcid.org/0000-0001-6045-562X

Address of the corresponding author:
Alp Ayan, T.C. İstanbul Kültür University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, 34156, Ataköy, Istanbul, Türkiye
E-mail: a.ayan@iku.edu.tr

Supplementary material

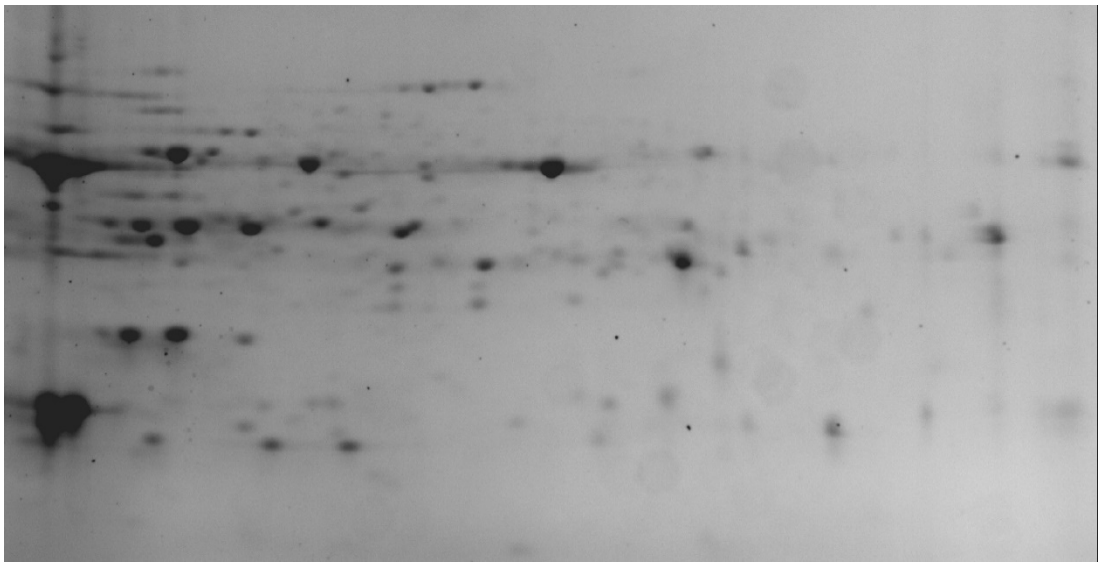

Fig. S1: Protein profiles of untreated S04-05 soybean variety.

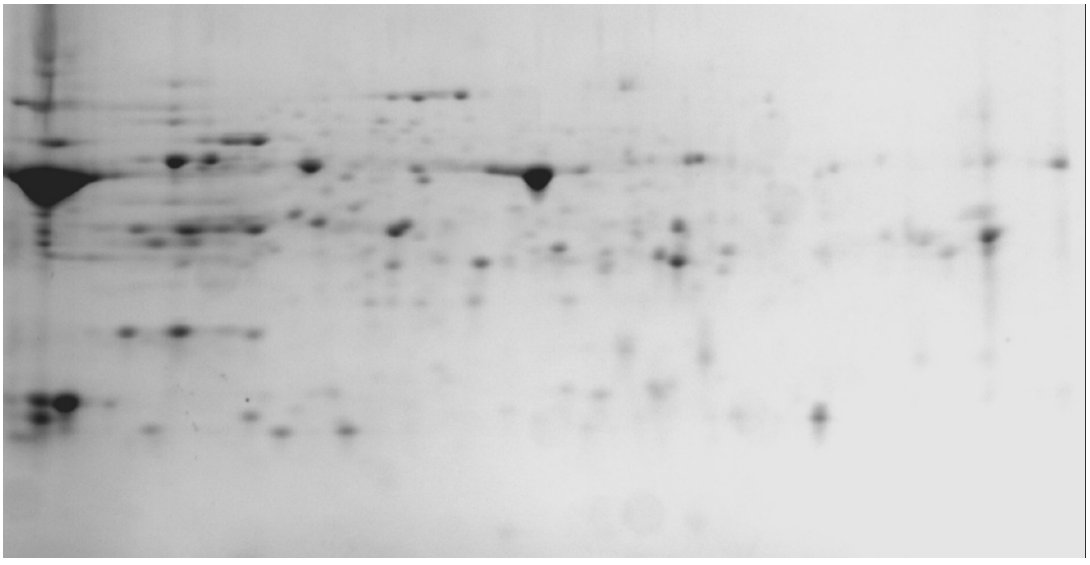

Fig. S2: Protein profiles of S04-05 soybean variety drought treated for 5 days.

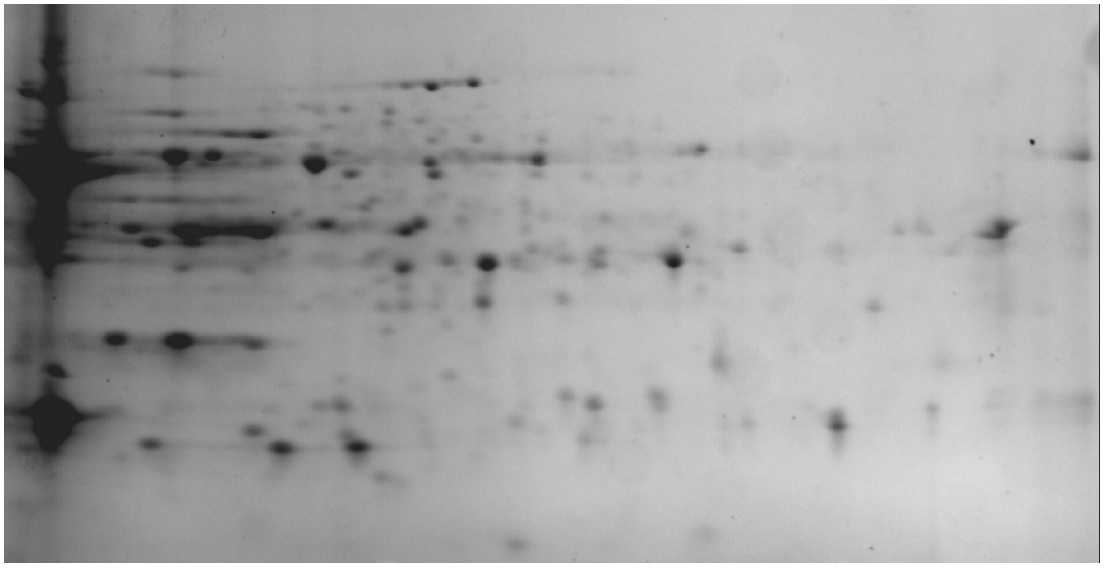

Fig. S3: Protein profiles of untreated SM1 soybean mutant.

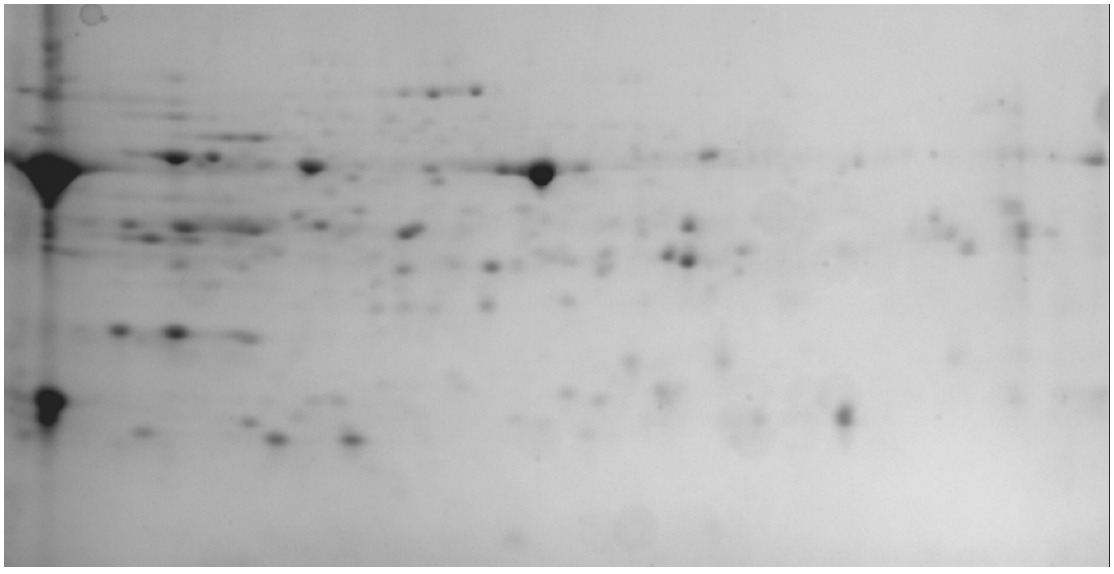

Fig. S4: Protein profiles of SM1 soybean mutant drought treated for 5 days

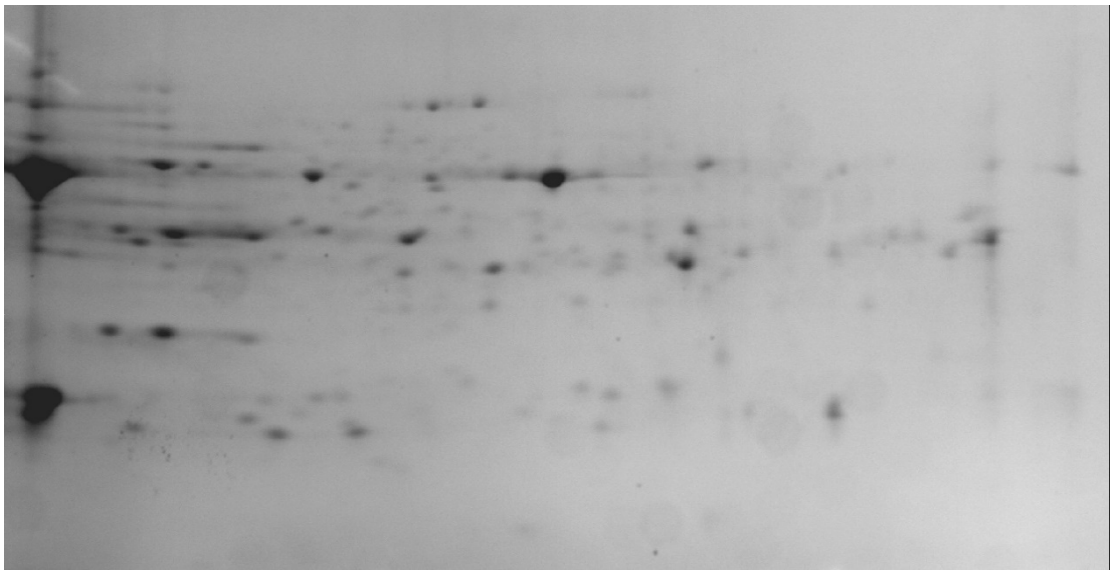

Fig. S5: Protein profiles of untreated SM3-1 soybean mutant.

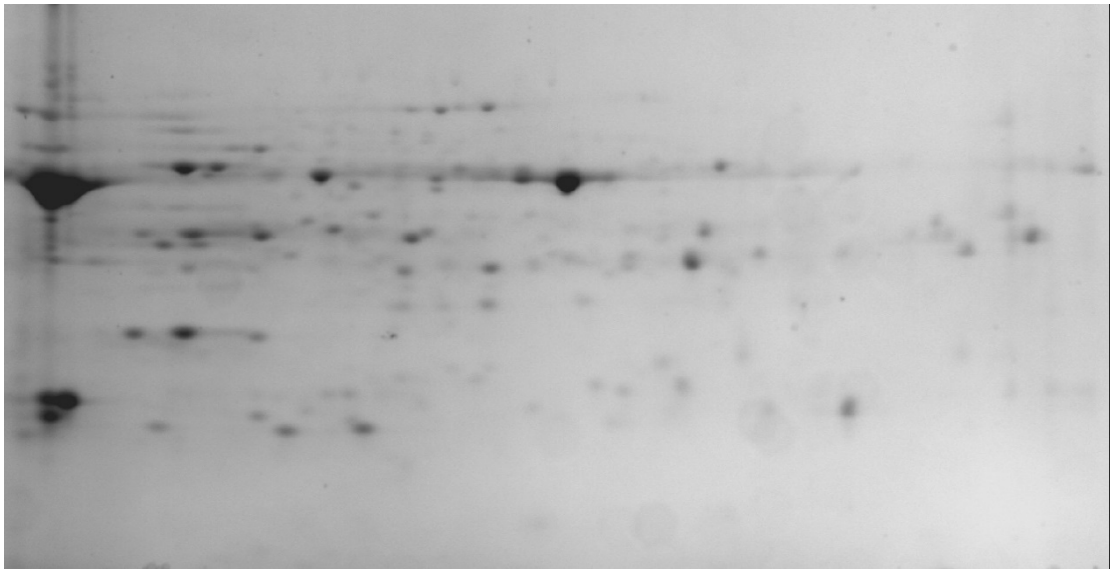

Fig. S6: Protein profiles of SM3-1 soybean mutant drought treated for 5 days.

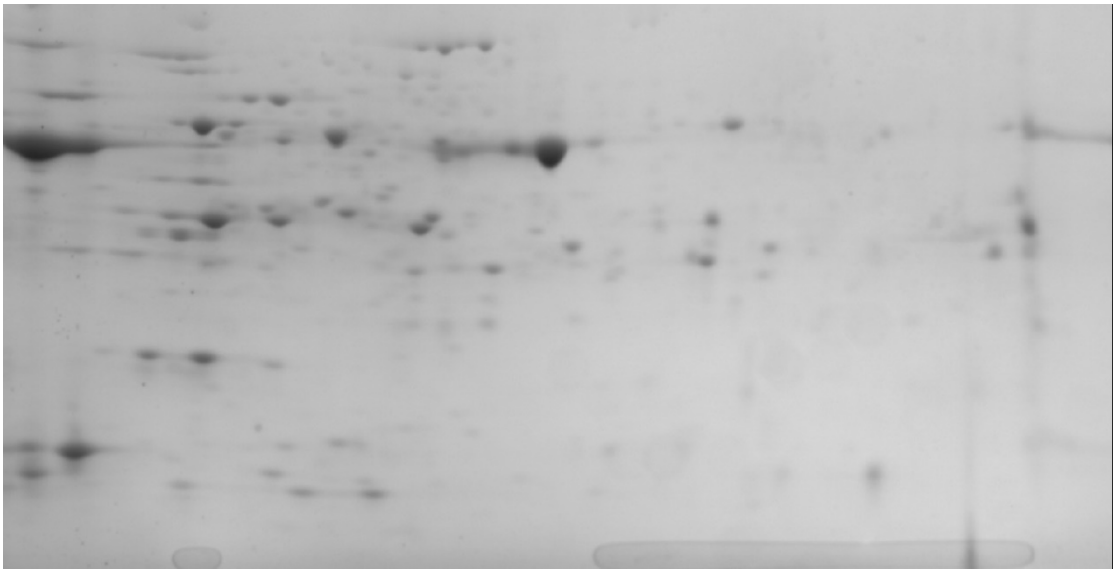

Fig. S7: Protein profiles of untreated S04-05 soybean variety.

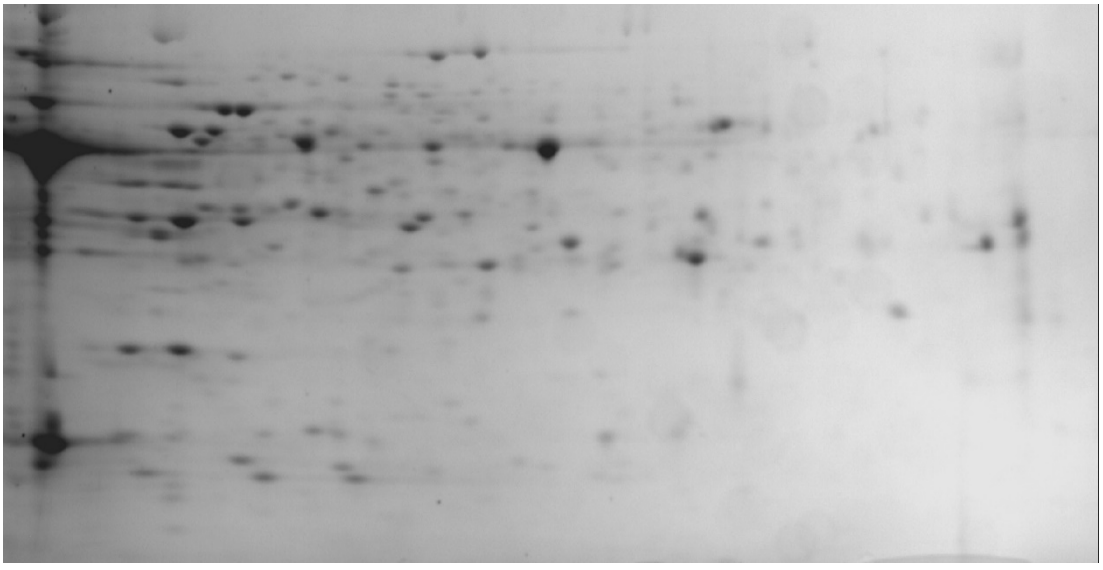

Fig. S8: Protein profiles of S04-05 soybean variety salt treated for 7 days .

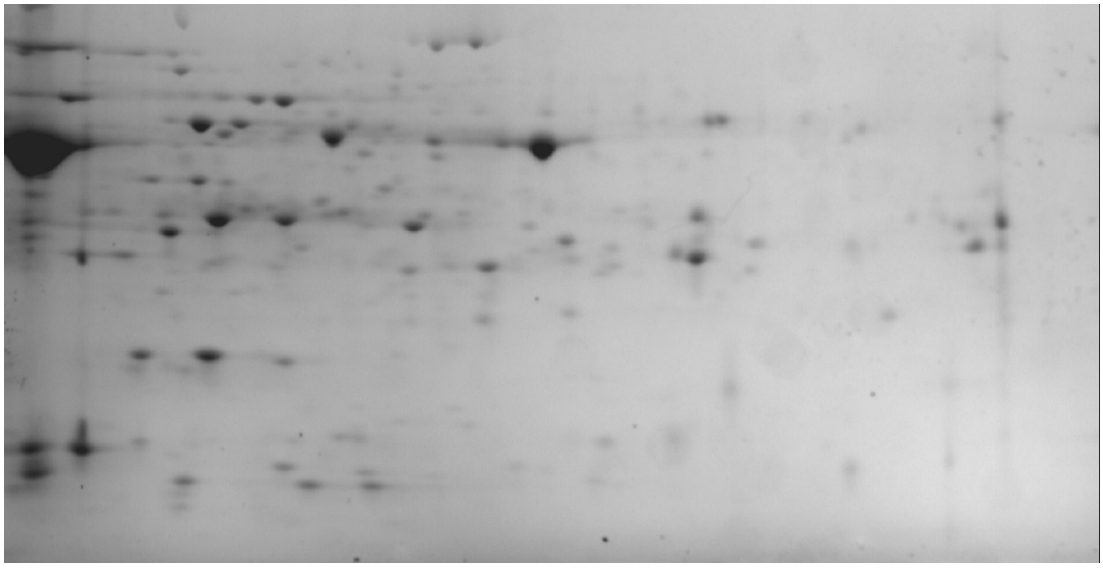

Fig. S9: Protein profiles of untreated SM1 soybean mutant.

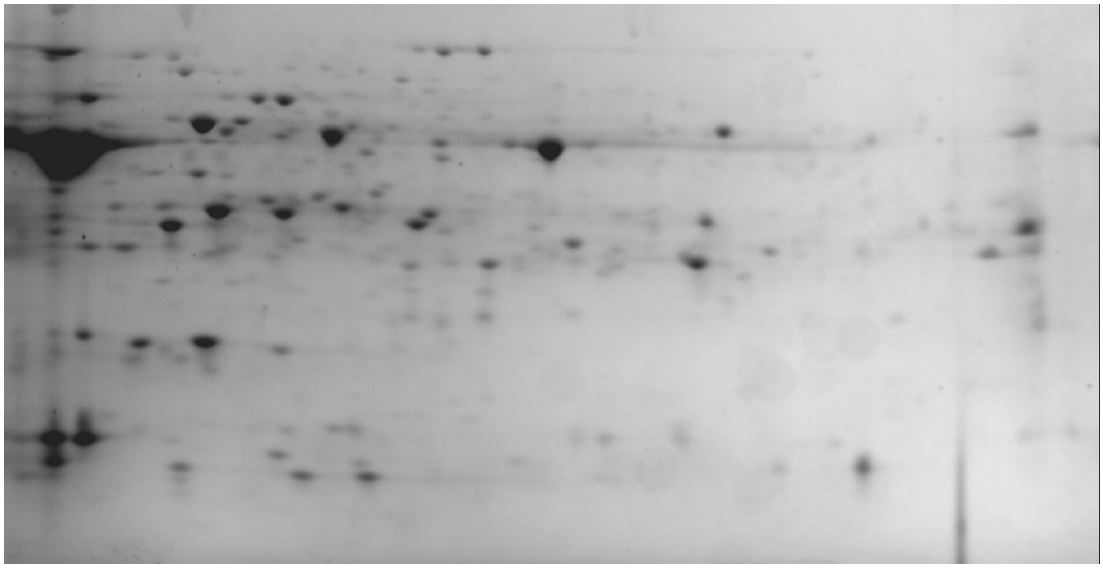

Fig. S10: Protein profiles of SM1 soybean mutant salt treated for 7 days.

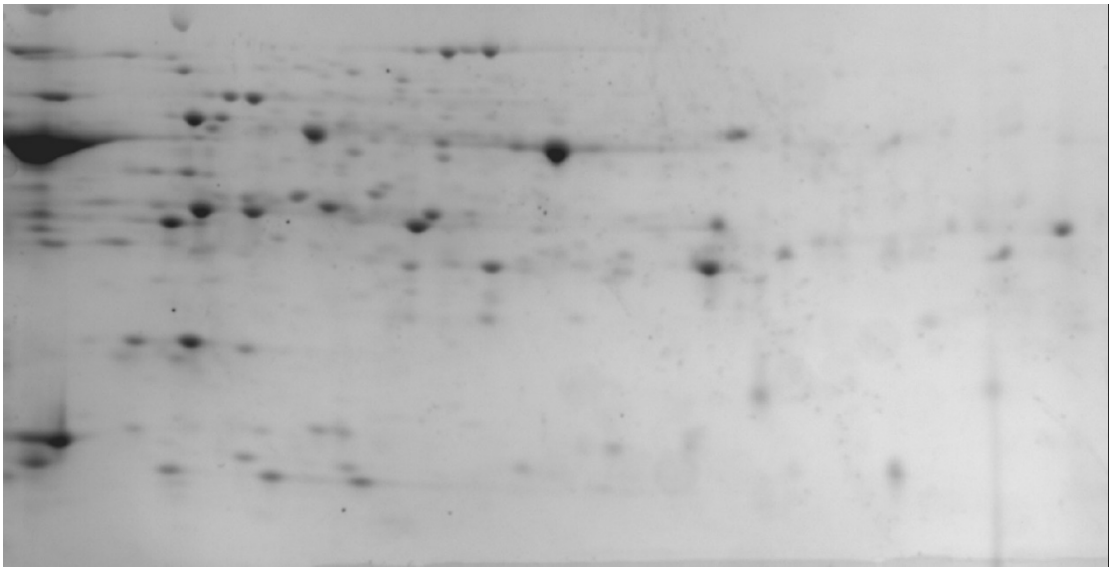

Fig. S11: Protein profiles of untreated SM3-1 soybean mutant.

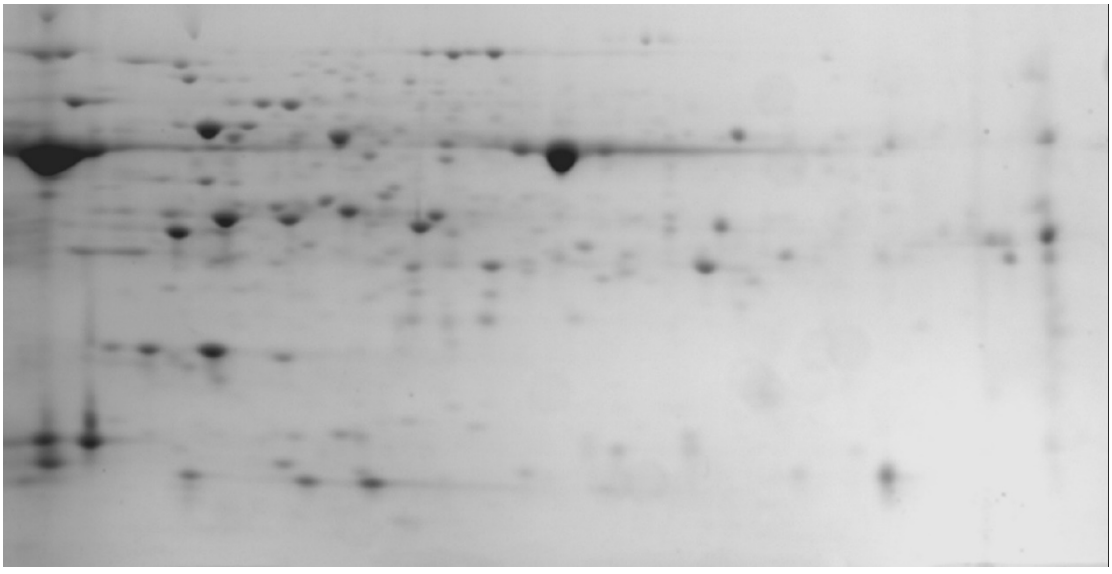

Fig. S12: Protein profiles of SM3-1 soybean mutant salt treated for 7 days.