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Differential Expression of RuBisCO large Subunit and its Assembly in Intact Chloroplasts of *Pisum sativum* L. Treated with NaCl and Jasmonic Acid

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THIS STUDY aimed to detect the effect of stress conditions imposed by different treatments of NaCl and jasmonic acid (JA) on the expression pattern and assembly of RuBisCO large subunit (RbcL) and RuBisCO enzyme complex using SDS-PAGE and HDN-PAGE techniques, respectively. Both Total Soluble Proteins (TSPs) and Chloroplast Proteins (ChPs) were variably affected by each treatment. Notably, a unique protein band was detected in the seedlings treated with 150 mM NaCl for 5 days. Surprisingly, this band was not detected under any other salt treatment. Using nano HPLC-ESI-MS/MS MALDI-TOF protein sequencing, this band was identified as downshifted RbcL at approximately 50 kDa in addition to the original one was separated at approximately 55 kDa on SDS-PAGE. The expression of RbcL protein decreased in a dose dependent manner in the plants treated with JA for one and five days. All treatments did not affect the RuBisCO complex assembly at approximately 660 kDa, but triggered stress-induced ChPs at approximately 440 kDa and 1 MDa concomitant with NaCl treatments. RbcL band isolated from intact chloroplast was associated with Coupling Factor (CF1) beta subunit of ATP synthase as revealed by MALDI-TOF protein sequencing analysis. Possible interpretations of these findings have been further discussed in this study.

Keywords *Pisum sativum* L., RuBisCO large subunit, Histidine Deoxycholate Native (HDN)-PAGE HDN- PAGE, NaCl stress, Jasmonic acid.

Introduction

Environmental stresses are one of the main constraints on crop performance. Variation in environmental signals is often unpredictable and need to be integrated with changes in gene expression patterns. Salinity causes up to US 12 billion dollars in agriculture losses every year (Flowers, 2004). Sobhanian et al. (2010) reviewed the effects of salt stress on several plants, including rice, soybean, wheat, potato and *Aleuropus lagopoides* and stated that reducing photosynthesis was a common response among them. Photosynthesis takes place in chloroplasts which are highly sensitive to salinity and hormonal changes (Biswal et al. 2008 and Saravanavel et al., 2011). Thus, protein analysis of stressed chloroplasts is important to understand the mechanism of tolerance (Rodziewicz et al. 2014). Abiotic stresses could negatively affect the activity of RuBisCO (EC.4.1.1.39) (Ratnakara & Rai, 2015). RuBisCO exhibits low catalytic rate, high competition with oxygenase activity and is considered a major target to enhance photosynthesis in plants (Parry et al., 2003).

In higher plants, synthesis and assembly of functional RuBisCO requires communication between the nucleus and chloroplast. RuBisCO small subunit (RbcS) is encoded by the nuclear genome, synthesized in the cytosol and transported to chloroplasts to bind with RuBisCO large subunits (RbcL) which are encoded by the chloroplast genome (Spreitzer & Salvucci, 2002). Catalytic properties of the enzyme are determined by the large subunit, which contains the active sites (Kasai et al., 1997). On the other hand, JA is a plant hormone recognized as being a signal during salinity stress (Yoon et al., 2009), involved in the control of gene expression (Yan et al., 2013 and Ghanati & Bakhtiarian, 2014), and recently recognized as senescence inducing

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signal throughout the plant kingdom (Liu et al., 2015). This action is congruent with earlier studies (He et al., 2002; Schommer et al., 2008 and Shan et al., 2011) in demonstrating that JA is involved, as a plant hormone, in defense responses, growth, development as well as leaf senescence.

Little is known about how the levels and patterns of RuBisCO expression are modulated by multiple dynamic abiotic factors such as NaCl and JA. The differential expression of RuBisCO enzyme and its assembly in chloroplast could be used to monitor stress effects (Saibo et al*.,* 2009; Khalifa & El Ghandoor, 2011 and Ratnakara & Rai, 2015). This study aims to get more insights about possible effects of salinity and JA on the expression pattern of RbcL and the assembly of RuBisCO complex in garden pea seedlings.

Materials and Methods

Plant material

Seeds of garden pea plants (*Pisum sativum* L. cv. Master B) were provided by the Agricultural Crop Institute, National Agriculture Research Center, Giza, Egypt to be used as a model plant in this study. Viability percentage was tested and recorded as 99 % for the whole seed batch to assure homogenous germination and confirm their vitality.

Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (Munich, Germany), VWR (Darmstadt, Germany), and Roth (Karlsruhe, Germany). Digitonin (Dig) and CoBB (G-250) were obtained from SERVA (Heidelberg, Germany). n-Dodecyl β-D-maltoside (DDM) was purchased from Sigma Aldrich (Munich, Germany). HDN-PAGE protein molecular weight standards from GE Healthcare (Freiburg, Germany). Protease inhibitors E64 and AEBSF were purchased from VWR (Darmstadt, Germany) and L-Histidine from Roth (Karlsruhe, Germany). Protease inhibitor cocktail complete (without EDTA) was ordered from Roche (Deutschland, Germany). Jasmonic acid (JA) was purchased from Chayman (USA). The MS media was purchased from Caisson Labs (USA). All chemicals used in this study were of analytical pure grade.

Seed germination and seedlings exposure to NaCl and Jasmonic acid treatments

Seeds of equal shape and color were manually selected, washed and soaked in drop wise running water for 24 h to induce homogenous germination. Hundreds of seeds were used for each treatment and each experiment was repeated 3 times to make sure that the results were reproducible. Seeds were surface sterilized by washing in 70 % ethyl alcohol and then immersed in 20% bleach solution (20 Clorox: 80 distilled water), shacked well and incubated for 15 min. Seeds were washed 4 times with sterile distilled water before being plated on MS media (Murashige & Skoog, 1962) containing 0.8% agar, 0.4% MS, 0.097% MES buffer and adjusted to pH 5.7 using potassium hydroxide and supplemented with either 50, 100, 150, 200, 300 or 400 mM of NaCl for 1, 3, 5 and 7 days. Regarding JA treatment, pea seeds were surface-sterilized and subjected to different regimes of JA treatments. During regime I, pea seeds were soaked in 1 µM jasmonic acid for 4, 8 and 12 h, plated on 0.8% agar media, and left for germination for three weeks. Samples were then harvested to be fractionated by SDS/HDN-PAGE techniques. In regime II, pea seeds were plated on 0.8% agar media containing 0.5 and 10 µM of JA, left for germination for 3 weeks, and then samples were collected to be analyzed by SDS/HDN-PAGE techniques. In regime III, pea seeds were plated on 0.8 % agar germination media for five days in dark. After the plumule emerged, pea seeds were grown in long light conditions (16 h light/8 h dark) for two weeks and transferred to MS treatment media (supplemented with 0.5, 5 and 10 µM of JA) for 1, 3 and 5 days, then samples were collected to be analyzed by SDS/HDN and HDN –PAGE echniques. All steps were carried out under sterile conditions using laminar airflow hood and filter sterilized JA.

Protein extraction

Proteins were extracted immediately after seedling collection according to Khalifa & El-Ghandoor (2011). Briefly, seedlings were ground in liquid N_2 and homogenized in Trizma buffer pH =8 (W/V) (Sigma- Aldrich, St. Louis, MO, USA); 0.1 mM phenylmethyl sulfonylfluroide (PMSF) as a protease inhibitor and 100 mM dithiothreitol (DTT) as a reducing agent (instead of mercaptoethanol). All extraction steps were done at 4°C. Total soluble proteins were collected by centrifugation at 12,000 rpm for 30 min. The supernatant was mixed with activated charcoal to eliminate pigments. Protein concentrations of different samples were measured spectrophotometrically using Pierce protein assay kit (thermo science, USA) in relation to BSA (Bovine Serum Albumin) standard.

Chloroplast isolation and protein extraction

Chloroplasts were isolated from young green leaves after the course of treatment before sunrise. All extraction steps were performed at 4°C following the method of Ladig et al. (2011). The respective amount of digitonin (final 1-1.5 %) and DDM (final 1 - 2 % (w/v), then water was added to a final volume of 40 µl. Finally, the ice-chilled buffer was supplemented with the membrane preparations. Solubilized samples were incubated for 30 min on ice and subsequently centrifuged for 30 min at 50 000 g to remove non-solubilized material. The supernatant was supplemented with a 5 % (w/v) Coomassie Blue G-250 in 0.5 M 6-aminohexanoic acid solution to set a detergent/Coomassie ratio of 8 g/g.

Fractionation of TSPs by SDS-PAGE

Denaturing gel was prepared of polyacrylamide in the presence of Sodium Dodecyle Sulphate (SDS-PAGE). Stacking gel (4% polyacrylamide) and resolving gel (12% polyacrylamide) were prepared according to Laemmli (1970). Equal amounts of protein extracts were loaded onto the gel (~20 µg / lane). Electrophoresis was performed at 45 mA onto an omniPAGE Mini Wide vertical slap (Cleaver scientific. Ltd, UK) following the manufacturer recommendations. The bands were stained over night with Coomassie brilliant blue (CoBB) G-250 solution then destained using the same solution without CoBB until the gel background became stain free. Gel images were captured using gel documentation system. Native gel was prepared according to Ladig et al. (2011).

Fractionation of ChPs by Histidine Deoxycholate Native (HDN)-PAGE

The reagents used in this method were: sample buffer (100 mM Tris-base, 0.5 M amino-hexanoic acid (ACA), 10% glycerol, 5 mM dithiothreitol (DTT), 2 mM $CaCl_2$, 1 mM aminoethyl benzene sulfonyl fluoride hydrochloride (AEBSF), 10 µM E64, 0.1 U/ µl micrococcal nuclease, and 2% digitonin (as a detergent); cathode buffer (3 mM Tris-base, 100 mM histidine base, 0.05% Sodium deoxycholate (Na-DOC) and 0.01% dodecyl maltoside (DDM)); anode buffer (100 mM Tris base); gel buffer (200 mM Tris base and glycerol (10-20%)); lower gel (2 M tris base, 3.5 % Acrylamide, 0.1% glycerol, 0.01% TEMED, 7% ammonium persulfate (APS)) and higher concentration gel (2 M Tris base, 12% acrylamide, 45% glycerol, 1% APS and 0.08% TEMED). All chemicals and reagents were purchased from Sigma Aldrich, USA.

Sample preparation for MALDI analysis

The protein bands were sent to proteome factory AG (Berlin, Germany) for identification by nano HPLC-ESI-MS/MS. The system consisted of 1100 nano HPLC (Agilent, Waldbronn, Germany), electrospray emitter (New objective, Woburn, MA) and an orbitrap XL or LTQFT ultra mass spectrophotometer (Thermofisher Scientific, Bremen, Germany). Protein bands were in gel digested with trypsin (Promega, Mannheim, Germany) then peptides were first trapped and desalted on the enrichment column (Zorbax 300 SB-C18, 0.3 x5 mm Agilent) for 5 minutes in the presence of a solvent (2.5% acetonitrile/0.5% formic acid) then separated in a zorbax 300 SB-C18 (75 µm x 150 mm column (Agilent)) using linear gradient from 10% solvent A 32%/ solvent B (A: 5% acetonitrile in water; B: 5% acetonitrile in 0.1% formic acid). Ions of interest were subjected to MS/MS. Samples were analyzed using TOF-TOF 4800 Maldi Analyzer from ABSciex in reflector positive mode. Six peptides mixture from ABSciex was used for calibration of MS and MSMS mode. Automated acquisition of MS data followed by MS/MS data was controlled by 4000 Explorer software. An acceleration voltage of 20 kV was used in MS reflector mode and 8 kV in MSMS mode.

Database search for protein identification

The protein search was performed using Protein Pilot 4.0 software from ABSciex against garden pea data base. This software incorporates the Paragon™ data base search engine, using the Pro Group™ algorithm for results compilation. The minimum acceptance criterion was 99% confidence level. The protein identification was done based on search against ITAG2.3 data base available on mascot search engine (Matrix Science, London, England). Only peptides score 20 or above were considered.

Results and Discussion

Effect of salinity and JA treatments on seedlings' growth

Three week-old pea seedlings grown on media supplemented with 50,100,150, 200, 300 and 400 mM NaCl for 1, 3, 5 and 7 days showed variation in their morphology (Fig. 1, panels A-D). Those treated with 150 mM NaCl or lower showed normal growth and morphological aspects as the control plants. On the other hand, plants grown on 200 mM NaCl and higher concentrations showed the first sign of wilting at the fifth day of seedlings growth (Fig. 1, panels C, arrowheads).

Moreover, necrosis and partial leaf death were detected on the seventh day (denoted by arrows, Fig. 1, panel D). Notably, the length of the hypocotyl regions (denoted by dashed rectangles, Fig. 1, panel D) at 150 and 200 mM NaCl stress conditions recorded on the seventh day was much higher than the relevant epicotyl length of pea seedlings grown at earlier growth stages of lower NaCl concentrations. Bolting preceded the signs of wilting, necrosis, and partial leaflets death.

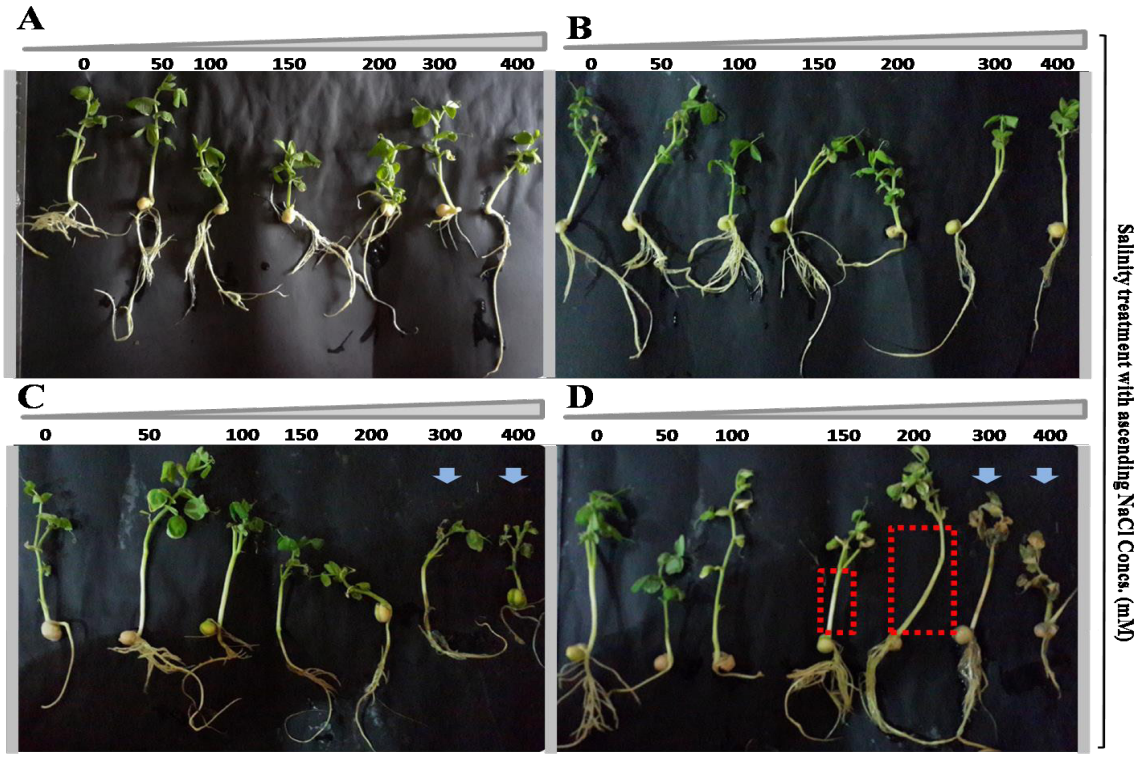

Fig. 1. Representative images showing the effect of different NaCl concentrations on 3-week-old garden pea seedlings grown on MS media for A: one day, B: three days, C: five days, and D: seven days.

Effect of salinity and Jasmonic acid treatments on TSPs banding patterns using SDS-PAGE

TSPs were extracted and fractionated from pea seedlings after two, three, five and seven days, using SDS PAGE (Fig. 2, panels A-D). Appearance of high molecular weight protein polypeptides, approximately 250 kDa especially at higher concentrations of NaCl treatments, were detected (denoted by arrow heads, Fig. 2, Panels A, B, and D). Also, a major protein band at approximately 55 kDa was detected in all NaCl treatments as well as the controls (Fig. 2, panels A-D). This band is known to represent RbCL protein in peas and others (Khalifa & El-Ghandoor, 2011). Thereby, protein band running at 55 kDa will be referred to RbcL throughout this study. Moreover, the intensity of RbcL. The intensity of this band was gradually increasing as NaCl concentration increased until a significant decrease was observed at the highest concentration of 400 mM NaCl (Fig. 2, panels B-D, lane 7). This result is similar to that of Ping et al. (2011) where salt treated *Arabidopsis* plants showed significant increase in RuBisCO biosynthesis. Interestingly, a new polypeptide band was detected at approximately 50 kDa in the plants treated with 150 mM NaCl for 5 days (Fig. 2, panels C, lane 4, denoted by arrowhead). This new band was identified by nano HPLC-MS/MS mass spectrometry as RbcL (Table 1) in addition to the normally present RbcL protein band separated at 55 kDa.

Plants treated with 200 mM NaCl or higher failed to form the newly modified RbcL at the fifth day. This may had directly and/or indirectly triggered necrosis and leaf death on the seventh day in the doses higher than 150 mM NaCl. RbcL undergoes many co- and post-translational modifications such as methylation

(Mininno et al., 2012). RuBisCO is methylated at the N-terminal methionine of RuBisCO small subunit (RbcS) and at the lysine-14 of RbcL in some species by large subunit methyle transferase (LSMT) enzyme which is present in all plant genomes. The methylation status is species-specific and does not affect the biological activity of RuBisCO (Trievel et al., 2003 and Lehtimäki et al., 2015). However, it is possible that binding/unbinding activity of RbcL is regulated by methylation. Interestingly, methylated RuBisCO is only present in *Pisum sativum*, *Nicotiana tabacum* and *Arabidopsis thaliana*, but not in corn, spinach, or wheat (Raunser et al., 2009). In pea plants, RbcL is tri methylated at lys14 and the molecular size of RbcL can change according to the methylation status (Raunser et al., 2009). On the other hand, the absence of lysine-14 methylation in spinach is likely due to the presence of monofunctional LSMT that can only methylate RbcS but not RbcL (Ying et al., 1999 and Mininno et al., 2012). It can be assumed that replacing hydroxyl by methoxy group during RuBisCO methylation will result in increasing the enzyme size, stability, hydrophobicity and decreasing its mobility (Scheiner et al., 2001 and Trievel et al., 2003). More hydrophobic RbcL may interact more with the chloroplast membrane without being able to pass due to the larger size and immobilization. Therefore, it could be suggested that 150 mM NaCl treatment is the threshold in that represents the maximum salt tolerance capacity that pea seedlings can cope with. Hence, it might be concluded that the 5 kDa downshift of newly identified band might be speculated initially as reprogramming of RbcL biosynthesis to adapt at this particular growth conditions.

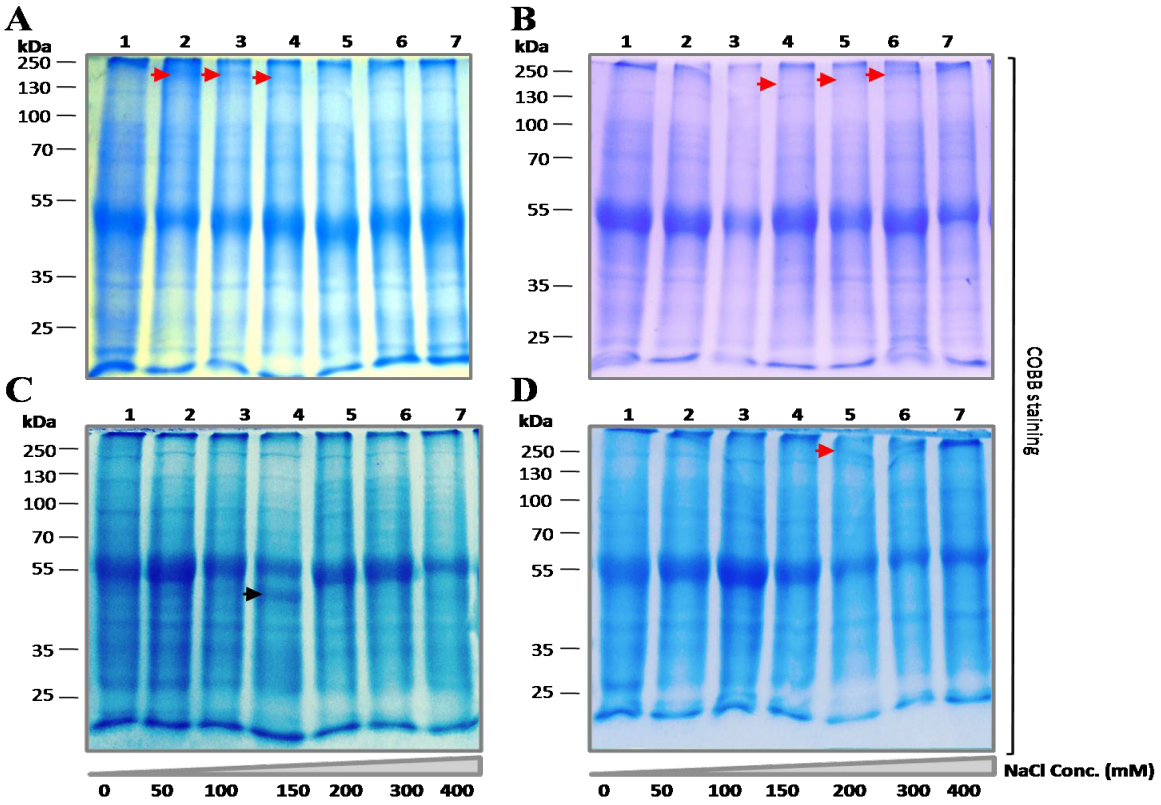

Fig. 2. Representative SDS-PAGE image showing the banding patterns of extracted TSPs from 3-week-old garden pea seedlings grown on MS media supplemented with 50, 100, 150, 200, 300, and 400 mM NaCl for A: one day, B: three days, C: five days, and D: seven days. Fermentas PageRuler™ Plus Prestained Protein Ladder (SM1811) was loaded and denoted by numbers left-handed of the figure indicating molecular weight standards in kDa.

TABLE 1. Analysis of selected *Pisum sativum* polypeptides by Mass spectrometry.

Spot	Polypeptide Type[a]	M.W[b]	M.W[c]	Name	Ion[d] score	Protein hits	Sequence coverage	MSC[d]
1	Extracted as Total soluble cellular proteins (TSPs)	~ 53 kDa	53.243	Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (LS) (chloroplast) [*Pisum sativum*]	42.70	gi|293338550	59 %	2025
2	Extracted as Total chloroplast proteins (ChPs)	~ 60 kDa	53.147	ATP synthase Coupling Factor (CF1) beta subunit (chloroplast) [*Pisum sativum*]	68.68	gi|293338551 gi|12148*	42 % 75 %*	2537

To manifest the effect of exogenous application of JA treatments on RuBisCO synthesis, JA treatments were applied in different strategies to either pea seeds or seedlings in this study. Our results showed that seedlings grown by strategy I and II (as described in the materials and methods section) did not show significant morphological differences or pronounced changes within TSPs content, especially at RbcL protein expression level (Fig. 3 I-II, panels A and B). However, leaves were more condensed at higher doses and tended to cluster together, as compared to the control in the first day of treatment (Fig. 3 II, denoted by red arrows). Unfortunately, the latter observation did not reflect or trigger any qualitative or quantitative changes in protein banding pattern (Fig. 3 I-II, Panel B). Unlike strategy I and II, strategy III did trigger several morphological and molecular tangible effects. For instance, new lateral shoot branching was observed in seedlings treated with 10 µM JA on the fifth day of JA application (Fig. 3 III, panel A). TSPs Fractionations exhibited a decrease in intensity of RbcL band with the applied JA concentration in one and five-days treatment (denoted by arrowheads, Fig. 3 III, panel B). The decrease in RbcL expression upon JA application was recorded before (Popova & Vaklinova, 1988). It had been shown that JA can induce the release of RuBisCO containing bodies (RCBs) from the chloroplast to the vacuole to be degraded (Chiba et al., 2003). In addition, degrading RbcL via RBCs is upregulated by JA application (Shan et al., 2011 and Lee et al., 2013) in a much higher rate than the decrease in the number of intact chloroplasts during senescence (Ono et al., 1995 and Adam et al., 2001). On another aspect regarding strategy III, quantitative changes of the expression levels of TSPs at approximate low molecular weight ranging from 15-35 kDa were detected in seedlings treated with 10 µM JA at the third days of JA application (Fig. 4 III, panel B, denoted by red arrowheads).

Effect of Salinity and JA treatments on total chloroplast proteins/protein complexes (ChPs) as revealed by HDN-PAGE

ChPs solubilized by 1.5 % digitonin SDS-PAGE fractions has revealed the presence of a significant new band at ~100 kDa (denoted by the upper arrowhead, Fig. 4, Lane 3) in plants treated with 150 mM NaCl for 5 days. This band was absent in all other treatments with either with JA or NaCl. Moreover, we observed a fluctuation

in protein expression separated at 60 kDA above and below he control level in NaCl and JA treated plants, respectively (denoted by the two lower arrowheads, Fig. 4, Lanes 2-3). This newly expressed band was identified by nano HPLC-MS/MS as chloroplastic ATP Synthase coupling factor 1 (CF1) beta subunit (Table 1).

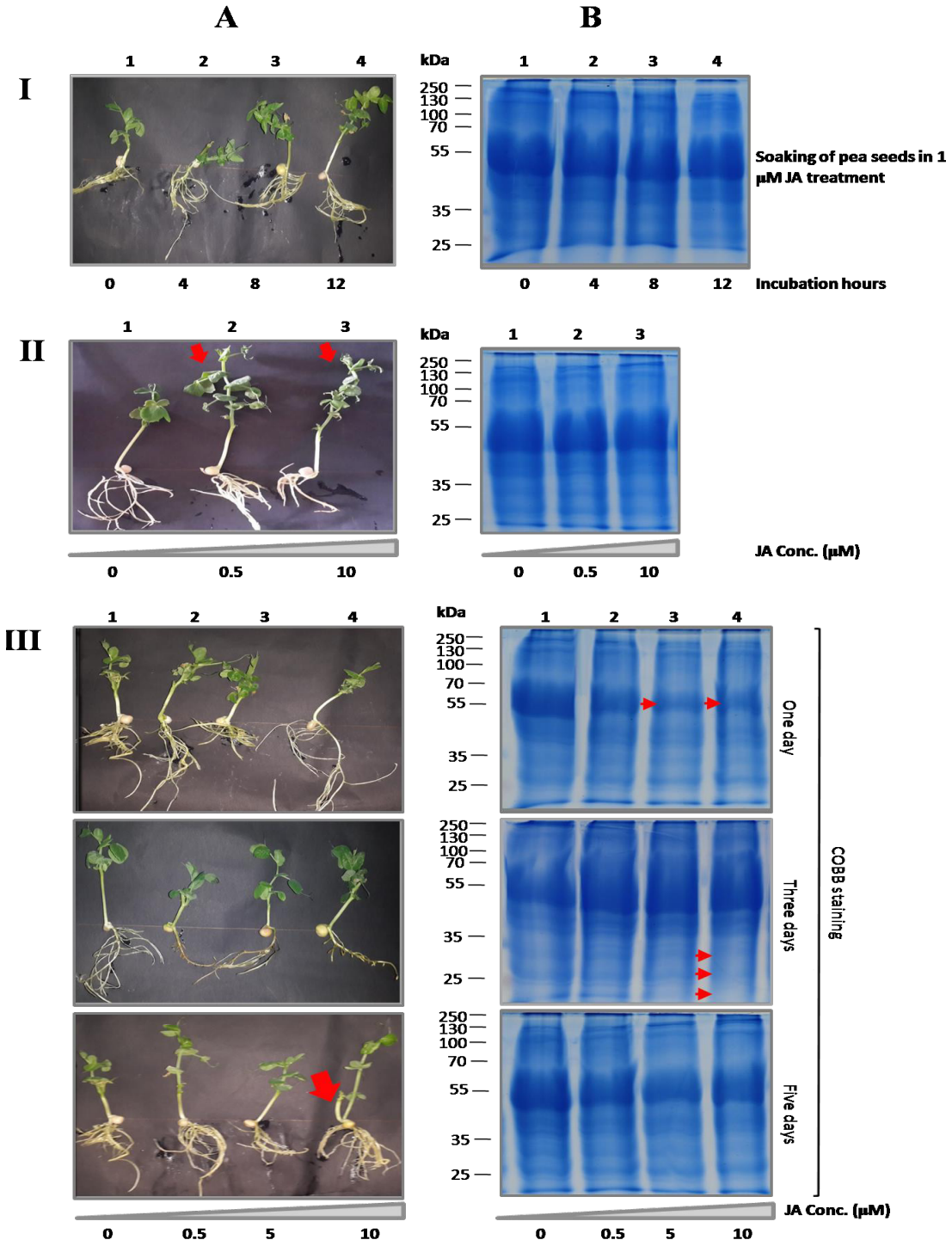

Fig. 3. Effect of different concentrations of JA treatments on the growth of garden pea seedlings grown on MS media supplemented with 0.5, 5, and 10 µM after application of three different regimes I, II, and III as shown in details in Figure 3. Panel A shows representative images showing seedlings growth affected by different JA concentrations. Panel B shows SDS-PAGE image showing the banding patterns under previously described JA treatments. Fermentas PageRuler™ Plus Prestained Protein Ladder (SM1811) was loaded and denoted by numbers left-handed of the figure indicating molecular weight standards in kDa.

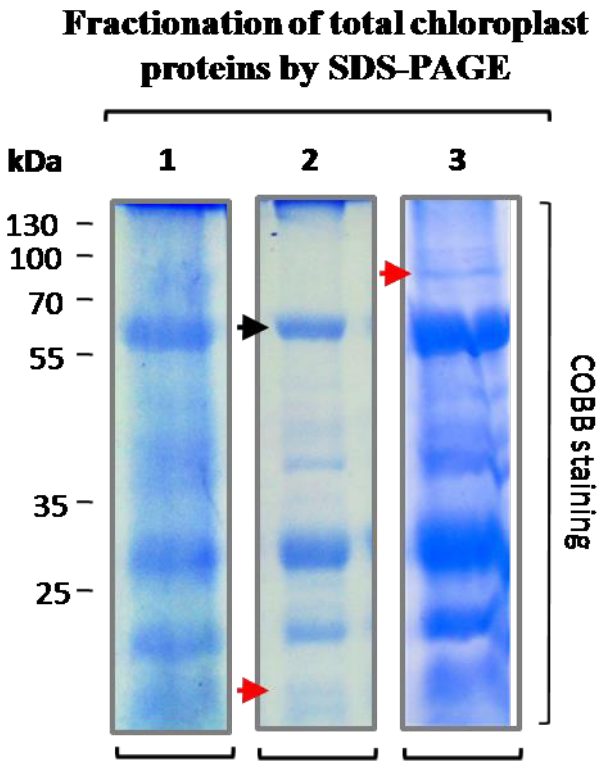

Fig. 4. Representative SDS-PAGE image of TSPs extracted from intact chloroplast of 3 week-old garden pea seedlings. Lane 1: untreated seedlings, lane 2: seedlings treated with 150 mM NaCl for 5 days, and lane 3: seedlings treated with 10 µM JA for one day. Fermentas PageRuler™ Plus Prestained Protein Ladder (SM1811) was loaded and denoted by numbers left-handed of the figure indicating molecular weight standards in kDa.

The best loading capacity and optimum detergent for HDN-PAGE solubilized ChPs are shown in Fig.5 (lanes 1-4). Solubilized pea chloroplasts by 1.5 % digitonin were detected as the best detergent percentage to solubilize RuBisCO enzyme complex. Therefore, 1.5 % digitonin-solubilized chloroplasts were used through this study to study the effect of high salt stress and JA treatments on pea seedlings under different fractionation conditions at high and low voltage (Fig. 6, panels A and B). In total, we observed 12 complexes in the range of 40 kDa to 1 MDa as estimated from the molecular mass standards. RuBisCO complex assembly at approximately 680 kDa was not affected on HDN-PAGE as compared to control (Fig. 6, panels A and B). However, differentially protein complexes were observed under and above RuBisCO complex at approximately 440 kDa and 1 MDa, respectively. This new band was absent in the control plants (denoted by stars, Fig. 6, panels A and B). The entity of these differentially expressed bands needs further investigation using protein sequencing approaches to increase our understanding of the mechanism of salt tolerance in plants.

In this work, RuBisCO expression was monitored at the cellular level (soluble RbcL in total cell lysate via SDS-PAGE) and organellar level (Assembly of RuBisCO complex using HDN-Native and RbcL using SDS-PAGE within chloroplast lysate). By this, RbcL expression was decreasing in TSPs fractionation level (Fig. 3) in response to JA treatment, while RuBisCO complex assembly did not reflect the same behavior in intact chloroplast (Fig. 6). In addition, RbcL resolved from intact chloroplast using SDS-PAGE was associated with coupling factor 1 (CF1) ATP synthase as revealed by mass spectrometry analysis. This association would indicate that the enzyme was regenerating and able to utilize ATP for its assembly likely via a chaperon protein (Kolesiński et al., 2011). However, this finding needs further investigation to determine the factor(s) controlling the assembly and degradation of RuBisCO.

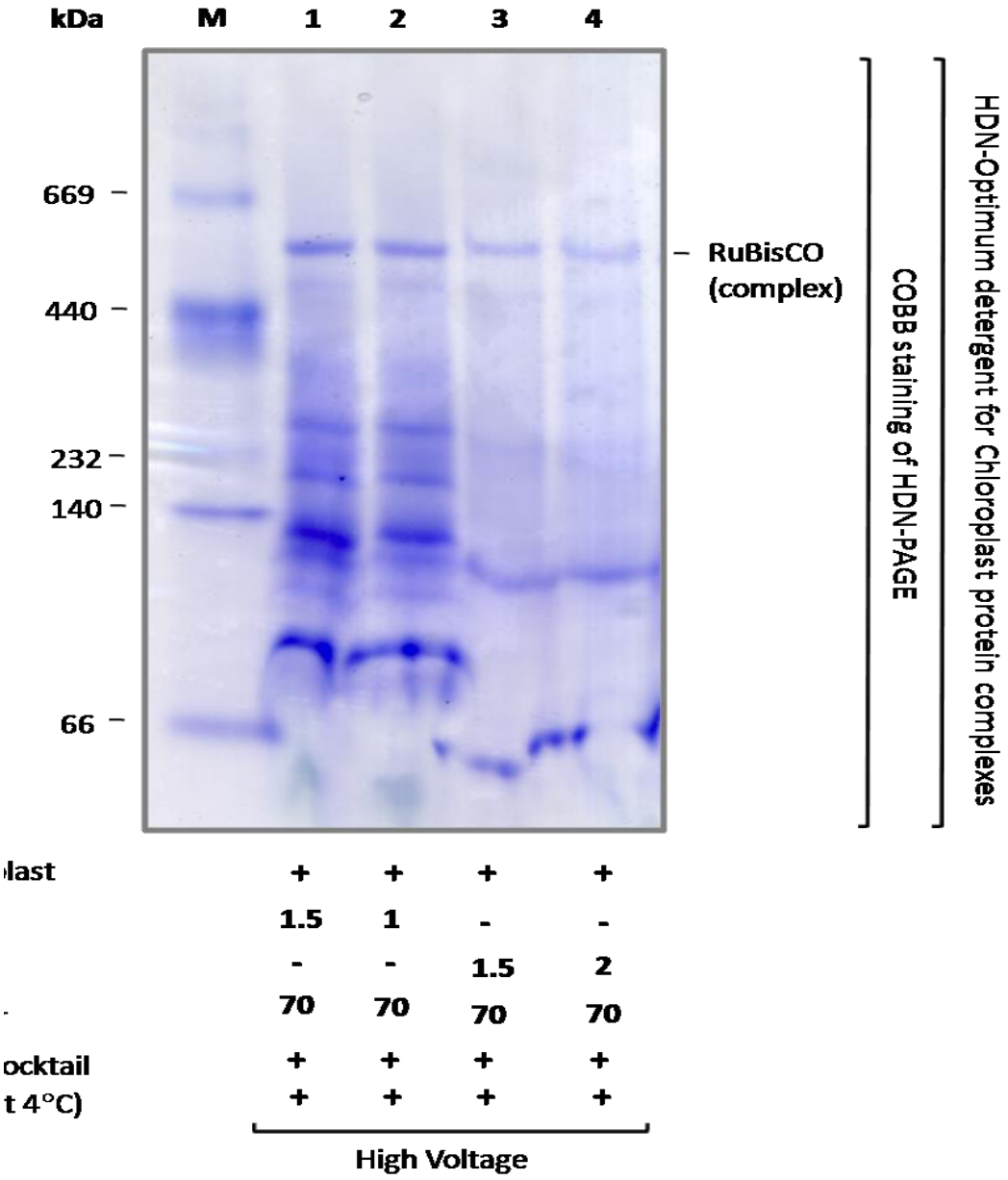

Fig. 5. Investigation of the loading capacity and optimum detergent for HDN-PAGE. Purified pea OEVs (30 µg) were solubilized by DDM using 1.5% (w/v) (lane 1) and 2% (w/v) (lane 2) and compared with the solubilized protein complexes pattern produced by digitonin (%w/v) using 1% (lane 3) and 1.5% (lane 4). Native molecular weight standards (HMW Native Marker kit, GE Healthcare) (lane M) were loaded and denoted by numbers left-handed of the figure indicating molecular weight standards in kDa.

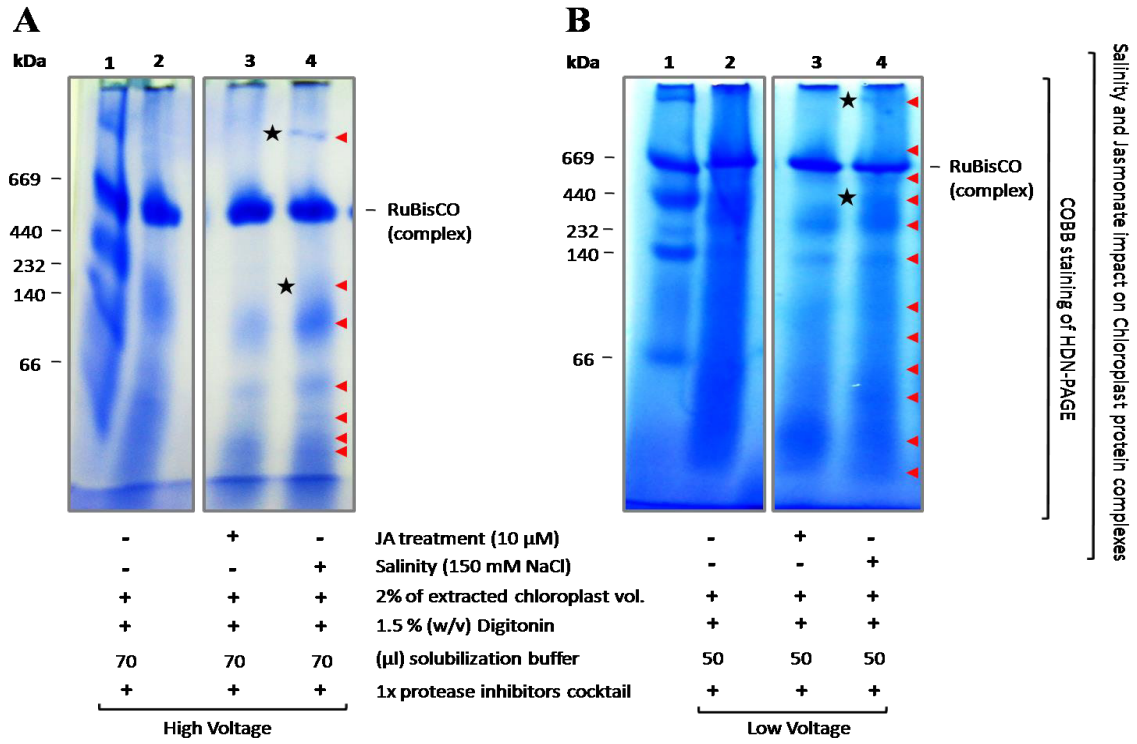

Fig. 6. Fractionation of ChPs by Histidine Deoxycholate Native (HDN)-PAGE technique from isolated intact chloroplasts of 3 week-old garden pea seedlings after salinity and JA treatments. Panel A: High voltage HDN-PAGE investigated solubilized pea intact chloroplasts with 70 µl (lanes 2-4). Panel B: Low voltage HDN-PAGE monitored solubilized chloroplasts with 50 µl (lanes 2-4). (+) means presence and (-) means absence of represented key parameters used in the optimization steps of HDN-PAGE technique. Native molecular weight standards (HMW Native Marker kit, GE Healthcare) (lane 1) was loaded and denoted by numbers left-handed of the figure indicating molecular weight standards in kDa. Lane 1: untreated seedlings, lane 2: seedlings treated with 150 mM NaCl for 5 days, and lane 3: seedlings treated with 10 µM JA for one day.

Analysis of selected Pisum sativum polypeptides by MALDI-TOF

Identified polypeptides were relevant to their corresponding marked protein polypeptides (black arrowheads) solubilized and separated by SDS-PAGE. The name of identified polypeptide (gene product) indicated gene annotation according to *Pisum sativum* L. database. **a:** Refers to isolated polypeptide either TSPs (spot 1; Fig. 2, panel C, lane 5) or ChPs (spot 2; Fig. 6, lane 3). **b:** represented approximate molecular weight of analyzed protein polypeptides in kDa as revealed by SDS-PAGE. **c:** Predicted molecular weight. *: This protein hit and the percentage of sequence coverage was identified and estimated according to NCBI BLAST search (see the discussion section). **d:** (MSC) stands for Mascot Search Score and ion score of digested identified polypeptides as revealed by Mascot protein identification software for Mass spectrometry data. The measurements obtained from two independent SDS-PAGE separations and subsequent MS analysis. Arrowheads point to analyzed bands after SDS-PAG.

Conclusion

The present study speculates that slightly higher concentration of NaCl (150 mM) may affect RuBisCO methylation, while JA affects its degradation via RCBs. Moreover, applied JA (0.5-1µM) enhaced the vegetative growth of pea seedlings. This study gives more insights about the inhibitory effect of environmental stress conditions on the assemblage of RuBisCO complex; firstly by expression of new form of RbcL subunit and secondly by derivation of stressed-induced supercomplxes (1MDa) and/ or RuBisCO-conjugated multiprotein complexes (440 kDA) that would recover or substitute

degraded RbcL subunits. In general, several pathways and regulatory mechanisms for RuBisCO assembly/degradation could be potentially tuning according to the applied stress condition.

References

Adam, Z., Adamska, I., Nakabayashi, K., Ostersetzer, O., Haussuhl, K., Manuell, A., Zheng, B., Vallon, O., Rodermel, S.R., Shinozaki, K. and Clarke, A.K. (2001) Chloroplast and mitochondrial proteases in Arabidopsis. A proposed nomenclature. *Plant Physiol.* **125**, 1912–1918.

Biswal, B., Raval, M.K., Biswal, U.C. and Joshi, P. (2008) Response of photosynthetic organelles to abiotic stress: modulation by sulfur metabolism. In:"*Sulfur Assimilation and Abiotic Stress in Plants*" Khan, N.A., Singh, S., Umar, S. (Ed.). pp. 167-191, Springer-Verlag, Berlin- Heidelberg.

Chiba, A., Ishida, H., Nishizawa, N.K., Makino, A. and Mae, T. (2003) Exclusion of ribulose-1,5-bisphosphate carboxylase /oxygenase from chloroplasts by specific bodies in naturally senescing leaves of wheat. *Plant and Cell Physiology,* **44**, 914–921.

Flowers, T.J. (2004) Improving crop salt tolerance. *J. Expr. Bot.* **55**, 307–319.

Ghanati, F. and Bakhtiarian, S. (2014)Effect of methyl jasmonate and silver nanoparticles on production of secondary metabolites by *Calendula officinalis* L (Asteraceae). *Trop. J. Pharmaceut. Res*. **13**(11), 1783-1789.

He, Y., Fukushige, H., Hildebrand, D.F. and Gan, S. (2002) Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. *Plant Physiol.* **128**(3), 876-884.

Khalifa, N.S. and El Ghandoor, H. (2011) Investigate the effect of Nd-Yag laser beam on soybean (Glycin max) leaves at the protein level. *Int. J. Boil.* **3**(2), 135-144.

Kasai, K., Nakamura, C., Mori, N. and Uchida, N. (1997) RuBisCO activity vs photosynthetic CO_2 assimilation rate in the alloplasmic hybrids of common wheat cv. Chinese Spring. *Wheat Inf. Serv.* **85,** 25–30.

Kolesiński, P., Piechota, J. and Szczepaniak, A. (2011) Initial characteristics of RbcX proteins from Arabidopsis thaliana. *Plant Mol. Biol.* **77,** 447–459.

Ladig, R., Sommer, M.S., Hahn, A., Leisegang, M.S., Papasotiriou, D.G., Ibrahim, M., Elkehal, R., Karas, M., Zickermann, V., Gutensohn, M., Brandt, U., Klösgen, R.B. and Schleiff, E. (2011) A high-definition native polyacrylamide gel electrophoresis system for the analysis of membrane complexes. *Plant J.* **67,** 181-197.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680 – 685.

Lehtimäki, N., Koskela, M.M. and Mulo, P. (2015) Posttranslational modifications of chloroplast proteins: An emerging Field. *Plant Physiol.* **168**(3), 768-775.

Lee, T.A., Wetering, S.W.V. and Brusslan, J.A. (2013) Stromal protein degradation is incomplete in Arabidopsis thaliana autophagy mutants undergoing natural senescence. *BMC Res. Notes.,* 6, 17. DOI: 10.1186/1756-0500-6-17

Liu, L., Li, H. and Zeng, H. et al (2016) Exogenous Jasmonic Acid and Cytokinin Antagonistically Regulate Rice Flag Leaf Senescence by Mediating Chlorophyll Degradation, Membrane Deterioration, and Senescence-Associated Genes Expression. *J. Plant Growth Regul*., **35**, 366. doi:10.1007/s00344-015-9539-0 .

Mininno, M., Brugière, S., Pautre, V., Gilgen, A., Ma, S., Ferro, M., Tardif, M., Alban, C. and Ravanel, S. (2012) Characterization of chloroplastic fructose 1,6-bisphosphate aldolases as lysine-methylated proteins in plants. *J. Biol. Chem.* **287**(25), 21034–21044.

Murashige,T. and Skoog, F.A. (1962) Revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum,* **15,** 473-497.

Ono, K., Hashimoto, H. and Katoh, S. (1995) Changes in the number and size of chloroplasts during senescence of primary leaves of wheat grown under different conditions. *Plant Cell Physiol.* **36,** 9–17.

Parry, M.A.J., Andralojc, P.J., Mitchell, R.A.C., Madgwick, P.J. and Keys. A.J. (2003) Manipulation of RuBisCO in the amount, activity, function and regulation. *J. Experiment. Bot.* **54,** 1321–1333.

Ping, L., Wind, J.J, Xiaoliang, S., Zhang, H., Hanson, J., Smeekens, S.C. and Teng, S. (2011) Fructose sensitivity is suppressed in Arabidopsis by the transcription factor ANAC089 lacking the membrane-bound domain. *Proc. Natl. Acad. Sci.* **108** (8), 3436–3441.

Popova, L.P. and Vaklinova, S.G. (1988) Effect of jasmonic acid on the synthesis of ribulose-1, 5-bisphosphate carboxylase-oxygenase in barley leaves. *Journal of Plant Physiology,* **133**(2), 210-215.

Ratnakara, A. and Rai, A. (2015) Influence of NaCl on the activity of ribulose 1,5-biphosphate carboxylase in three leafy vegetables. *Oct. J. Env. Res.* **3**(2), 163-166.

Raunser, S., Magnani, R., Huang, Z., Houtz , R.L., Trievel, R.C., Penczek, P.A. and Walz, T .(2009) RuBisCO in complex with RuBisCO large subunit methyltransferase. *PNAS,* **106**(9), 3160–3165.

Rodziewicz, P., Swarcewicz, B., Chmielewska, K., Wojakowska, A. and Stobiecki, M. (2014) Influence of abiotic stresses on plant proteome and metabolome changes. *Acta Physiolog. Plant,* **36**(1), 1–19.

Saibo, N.J.M., Lourenço, T. and Oliveira, M.M. (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. *Ann. Bot.* **103,** 609-623.

Saravanavel, R., Ranganathan, R. and Anantharaman, P. (2011) Effect of sodium chloride on photosynthetic pigments and photosynthetic characteristics of *Avicennia officinalis* seedlings. *Recent Res. Sci. Technol.* **3,** 177-180.

Shan, X., Li, C., Peng, W. and Gao, B. (2011) New perspectives of jasmonate function in leaf senescence. *Plant Signal Behav.* **6**(4), 575–577.

Scheiner, S., Kar, T. and Gu, Y. (2001) Strength of the $C^{\alpha}H..O$ hydrogen bond of amino acid residues. *J. Biol. Chem.* **276,** 9832–9837.

Schommer, C., Palatnik, J.F., Aggarwal, P., Chételat, A., Cubas, P., Farmer, E.E., Nath, U. and Weigel, D. (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. *Plos Biol.* **6**(9), e230. doi: 10.1371/journal.pbio.0060230

Sobhanian, H., Motamed, N., Jazii, F.R., Nakamura, T. and Komatsu, S. (2010) Salt stress induced differential proteome and metabolome response in the shoots of *Aeluropus lagopoides* (Poaceae), a halophyte C4 plant.

Spreitzer, R.J. and Salvucci, M.E. (2002) RUBISCO: structure, regulatory interactions, and possibilities for a better enzyme. *Ann. Rev. Plant Biol.* **53,** 449–475.

Trievel, R.C., Flynn, E.M., Houtz, R.L. and Hurley, J.H. (2003) Mechanism of multiple lysine methylation by the SET domain enzyme RuBisCO LSMT. *Nat. Struc. Biol.* **10,** 545 – 552.

Ying, Z., Mulligan, R.M., Janney, N. and Houtz, R.L. (1999) RuBisCO small and large subunit N- methyltransferases. Bi- and mono-functional methyltransferases that methylate the small and large subunits of RuBisCO. *J. Biol. Chem.* **274**(51), 36750-36756.

Yan, Y., Borrego, E. and Kolomiets, M.V. (2013) Jasmonate biosynthesis, perception and function in plant development and stress response. In:" *Lipid Metabolism*" Baez, R.V. (Ed.): . *Tech Rijeka,* Ch. 16. pp. 393–442. DOI: **10.**5772/52675.

Yoon, J.Y., Hamayun, M., Lee, S.K., Kyung, S. and Lee, I.J. (2009) Methyl jasmonate alleviated salinity stress in soybean. *J. Crop Sci. Biotech.* **12**(2), 63–68.

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التعبير الانتقائي لتحت الوحدة الكبرى و وحدة معقد إنزيم الروبيسكو (RuBisCO) فى نبات البسلة إثر معاملتها بكلوريد الصوديوم و حمض الجاسمونيك

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يعتبر إنزيم الروبيسكو (RuBisCO) من الإنزيمات الهامة التي تحدد كفاءة عملية البناء الضوئي، حيث يلعب دورا رئيسيا فى تثبيت الكربون. ويؤدي تفكك هذا الإنزيم إلى حدوث نقص في تثبيت الكربون و بالتالي قلة كفاءة عملية البناء الضوئى. وتهدف هذه الدراسة إلى رصد تأثيرات الإجهاد الملحى وهرمون النمو النباتى (الجاسمونيك) على مجموع البروتينات الخلوية وعلى التعبير الجينى لتحت الوحدة الكبرى (RbcL) لمعقد إنزيم (RuBisCO). باستخدام الفصل الكهربائي باستخدام هلام عديد الأكريلاميد (SDS-PAGE) لتنفيذ هذا. بالإضافة إلى ذلك فقد تم عزل وتنقية البلاستيدات الخضراء السليمة (غير المحطمة) لدراسة تأثير الظروف سالفة الذكر على مجموع البروتينات المعزولة من البلاستيدات الخضراء باستخدام (SDS-PAGE). كما تم أيضا فحص و دراسة سلامة/تفكك معقد إنزيم الروبيسكو باستخدام تقنية (Native PAGE) تحت تأثيرتركيزات مختلفة من الإجهاد الملحى ومعاملات مختلفة من هرمون الجاسمونيك. أيضا تم تسليط الضوء على مدى تغير مجموع المعقدات البروتينية بين العينات المعاملة وغير المعاملة. خلصت النتائج إلى تعيين بروتين خلوى بوزن كتلى 50 كيلو دالتون تحت تأثير الإجهاد الملحى بتركيز 150 mMوتم تعريفه باستخدام مطياف الليزر المحدد للكتل البروتيتنية (nano HPLC-ESI-MS/MS MALDI-TOF) بأنه تحت الوحدة الكبرى (RbcL) لمعقد إنزيم (RuBisCO) بالإضافة لتعيين بروتين تحت الوحدة الكبرى لمعقد إنزيم (RuBisCO) بوزن كتلى 55 كيلو دالتون. كما أظهرت الدراسة عن تعيين بعض المعقدات البروتينية (المعزولة من البلاستيدات الخضراء) بصورة انتقائية فى عينات الإجهاد الملحى بنفس التركيز الملحى السابق باستخدام مطياف الليزر لتحديد الكتل البروتينية . علاوة على ذلك فقد تم أيضا رصد اختلاف التعبير الجينى (على مستوى إنتاج البروتين) الخاص بـ Coupling Factor (CF1) beta subunit of ATP synthase فى العينات النباتية المعاملة بهرمون الجاسمونيك بتركيز 10 µM. وتخلص هذه الدراسة إلى أثر الإجهاد الملحى على سلامة/تفكك معقد إنزيم (RuBisCO) وباقى المعقدات البروتينية للبلاستيدات الخضراء، بالإضافة لجذب الانتباه لدراسة كافة السبل الممكنة لحماية هذا الإنزيم تحت ظروف النمو المجهدة أو/ و غير المناسبة.