



Research article

Proteomics unravel the regulating role of salicylic acid in soybean under yield limiting drought stress

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ABSTRACT

Drought is a major concern for sustainable yield under changing environment. Soybean, an economically important oil and protein crop, is prone to drought resulting in yield instability. Salicylic acid (SA), a multifaceted growth hormone, modulates a series of parallel processes to confer drought tolerance thereby relieving yield limitations. The present study was performed in soybean plants treated with SA (0.5 mM) through seed pre-treatment under drought regimes: severe stress (50% RWC) and moderate stress (75% RWC), and rehydration. Differential leaf proteome profiling with morphological, physiological and antioxidative metabolism studies were performed at two developmental stages (vegetative and flowering). This explained the tolerance attribution to soybean throughout the development attaining yield stability. **Abundance of proteins involved in photosynthesis and ATP synthesis generated energy driving metabolic processes towards plant growth, development and stress acclimation.** Carbon (C) metabolism proteins involved in growth, osmoregulation and C partition relieved drought-induced C impairment under SA. Defensive mechanisms against redox imbalance and protein misfolding and degradation under stress were enhanced as depicted by the abundance of proteins involved in redox balance and protein synthesis, assembly and degradation at vegetative stage. Redox signaling in chloroplast and its interplay with SA signaling triggered different defense responses as shown through thioredoxin protein abundance. Amino acid metabolism proteins abundance resulted in increased osmoprotectants accumulation like proline at initial stage which contributed later towards N (nitrogen) remobilization to developing sink. At later stage, abundance of these proteins maintained redox homeostasis and N remobilization for improved sink strength. The redox homeostasis was supported by the increased antioxidative metabolism in SA treated plants. The downregulation of proteins at flowering also contributed towards N remobilization. Yield potential was improved by SA under drought through acclimation with enhanced N and C remobilization to sink as demonstrated by increased yield parameters like seed number and weight per plant, thousand seed weight and harvest index. The potential of SA in conferring drought tolerance to plants to maintain sustainable yield possess future research interests.

1. Introduction

About 80% of the world's agricultural land is rain fed and under the threat of drought. The drought related yield reductions for the major crops in world will reach more than 50 per cent by 2050 (Li et al., 2009). The negative impact of water stress on agricultural productivity will make it challenging to meet the food demands of growing global population. In India, 64% of the population depends on agriculture for their livelihood. The country faces major challenges to increase its food production to attain 50% more grain by 2020 for its ever-growing

population (Kumar and Gautam, 2014). In India, about 68% area out of net sown 140 million hectares is vulnerable to drought conditions where about 50% area has frequent droughts. India has experienced large scale droughts and the frequency is increasing posing a great threat to agriculture and food security. Climate change will also impact water resources thereby posing more risks to agriculture in India as water is the most critical agricultural input.

Soybean [*Glycine max* (L.) Merr.] is the most important leguminous crop worldwide for essential source of oil, protein, macronutrients and minerals (Clemente and Cahoon, 2009). The predicted climate change

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with persistent droughts is a great threat to sustainability of soybean yields (Foyer et al., 2016). India has experienced increase of about 24% in drought with statistically significant trends in the spatial extent of droughts in Central Northeast and West Central regions and about 55% increase in the risk of severe drought (Mishra and Liu, 2014; Sharma and Mujumdar, 2017). India has about 56% of the net cultivated area as rain-fed accounting for 44% of food production. Rainfall is crucial for Kharif crops that accounts for about 70% oilseed production of the country (Crisis Management Plan Drought (National), 2017). Drought can cause 40–60% soybean yield loss worldwide (Valliyodan and Nguyen, 2006). Soybean production in India accounts for 4% of global production. Soybean is the source of vegetable seed oil (20%) and protein (40%) for human and also used in animal feed. Major soybean producing states are part of west central region of India which is facing severe drought. Bhatia et al. (2008) indicated 28% yield reduction in soybean under adverse soil moisture conditions in India. Between 26 and 34% of the yield variability in Indian soybean yields was explained by climate variability including drought (Ray et al., 2015).

The negative impact of drought predominates at all developmental stages, starting from germination to seed maturation (Valliyodan and Nguyen, 2006). Drought avoidance, drought tolerance, drought escape, and drought recovery are the mechanisms facilitating the plants to overcome stress (Cruz De Carvalho, 2008). Salicylic acid (SA), a plant growth hormone and important signaling molecule, has great agronomic potential to improve the drought tolerance of plants. SA modulates the plant responses to environmental stresses by regulating plant growth, development, ripening, and defense responses. Water deficit condition increased the level of endogenous SA upto fivefold in *Phillyrea angustifolia* (Munne-Bosch and Penuelas, 2003) and approximately twofold in barley roots (Bandurska and Stroinski, 2005). The role of SA in regulation of drought was also supported by the induction of SA-inducible genes *PR1* and *PR2* by drought stress (Miura et al., 2013). The *Arabidopsis* mutants *adr1*, *myb96-1d*, *siz1*, *acd6*, and *cpr5* accumulating endogenous SA exhibit SA-dependent drought tolerance (Chini et al., 2004; Seo et al., 2009; Miura et al., 2013). Lee et al. (2006) reported conferred drought tolerance in *Arabidopsis* on introduction of the pepper pathogen-induced gene *CAP12* accompanied by the expression of *Arabidopsis PR1* gene involved in SA induced defense responses. The application of low concentration of SA enhanced the plant growth and drought tolerance in wheat (Kang et al., 2012) and muskmelon (Korkmaz et al., 2007) under water stress. Senaratna et al. (2000) reported increased plant tolerance to drought, heat and chilling stress in tomato and beans by imbibition of seeds in 0.1–0.5 mM SA. Loutfy et al. (2012) reported increase in biomass, inorganic and organic solute contents of wheat under interactive effect of SA and drought. SA treatment increased the membrane stability and levels of proline and ABA in water stressed barley conferring plants with stress tolerance (Bandurska and Stroinski, 2005). SA positively influenced the ascorbate–glutathione cycle in pretreated wheat leading to enhancement in tolerance to stress and alleviating substantial water loss (Kang et al., 2013). The detrimental effects of water stress on photosynthesis were alleviated by SA pretreatment in wheat along with increased antioxidative metabolism (Singh and Usha, 2003). SA strengthened antioxidant defense system in *Zea mays* under drought stress (Saruhan et al., 2012). Molecular studies on SA induced genes under water stress demonstrated 9 highly expressed genes in guard cells including *LT130* (Miura et al., 2013). The over expression of *LT130* enhanced the expression of dehydrins involved in improving the drought tolerance. Proteomics revealed several different functionally characterized proteins to be upregulated by pretreatment with SA under drought stress in wheat (Kang et al., 2012). Several defence proteins such as glutathione S-transferase, ascorbate peroxidase and peroxiredoxin were upregulated suggesting the role of SA in protecting the plants from oxidative stress by enhancing the antioxidant defense system. SA increased the expression of ATP synthase to maintain the energy requirement for growth and coping with stress. Photosynthesis related proteins RuBisCO

and related enzymes were upregulated in wheat under treatment of SA and drought (Sharma et al., 2017).

SA is involved in the response to abiotic stress however the actual role of SA in abiotic stress remains unresolved. The present study will give an insight on the relationship between the SA and drought tolerance in soybean plants through physiological, biochemical and proteomics analyses. Subsequently, a network of different drought adaptive/resistant responses induced by SA in plants is proposed. We hypothesize that SA will improve the photosynthetic performance of soybean driving metabolic processes for stress acclimation to maintain growth and development under water limiting environment. Interplay of redox signaling and SA signaling is substantiated triggering defense mechanisms against drought. Yield limitations relieving role of SA under water stress will be revealed by analysis of different parameters related to nitrogen use efficiency (NUE) and yield.

2. Materials and methods

2.1. Biological material

The widely adaptable soybean (*Glycine max* (L.) Merr.) variety JS335 (JAWAHAR SOYBEAN 335) was selected for the experiment due to its good germinability and longevity. JS335 variety selected due to highest germination rate and adaptability to experiment site. The seeds were obtained from Indore, Madhya Pradesh, India.

2.2. SA application and plant growth conditions

SA application was performed through seed priming by soaking the seeds in 0.5 mM SA solution for 6 h before sowing and for control seeds were soaked in water. SA concentration was selected on the basis of highest seed germinability under SA pretreatment (results not shown). The experiment was conducted at CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh (26° 55' N latitude, 80° 59' E longitude and at an altitude of 113 m in subtropical climate). Recommended dose of NPKS (Nitrogen: Phosphorus: Potassium: Sulphur) at 20:60:20:20 kg per hectare was applied at the time of seed bed preparation. Seeds were sown at a depth of 2–3 cm adopting a spacing of 30 × 5 cm. Irrigation was maintained regularly till the seedling establishment.

Drought was maintained by controlling the irrigation after seedling establishment (45 days of germination) till the harvesting stage. The monitoring of the soil moisture level was performed on regular basis by Soil Moisture Meter (ICT International Pvt Ltd. Australia). The two stress levels were maintained on the basis of relative water content (RWC): 50% RWC (severe stress) and 75% RWC (moderate stress) with rehydrated plants after 50% RWC stress. The well watered, stressed and rehydrated control plants were also maintained for SA treatment.

CON = Control well watered; 50% = Control 50% stressed; 75% = Control 75% stressed; RH = Control Rehydrated.

CON + SA = SA treated well watered; 50 + SA = SA treated 50% stressed; 75 + SA = SA treated 75% stressed; RH + SA = SA treated Rehydrated.

RWC of the leaves was determined by following formula:

$$\text{RWC}\% = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \times 100$$

Here, FW = Fresh weight; DW = Dry weight; TW = Turgid weight of the leaf after equilibration in distilled water for 24 h.

2.3. Growth and yield

Plants were harvested in five replicates for biomass analysis at vegetative phase (8–9 weeks of growth), flowering stage (after heading initiation) and final harvest (full maturity). Leaf samples at both stages were freeze-dried in Liquid N₂ and store at –80 °C for further analysis. Root

and shoot biomass were weighed after drying the plants in oven at 80 °C for 96 h. Yield parameters were studied through number of seeds per plant, grain weight per plant, thousand grain weight and harvest index. Thousand grain weight, the weight of 1000 grains/seeds, used as measure of seed size and indicator of grain quality. Harvest index was calculated by following formula:

$$\text{Harvest Index} = \frac{(\text{Grain weight})}{(\text{Above ground biomass of plant})} \times 100$$

2.4. Physiological parameters

The light-saturated rate of CO₂ assimilation (A_N), stomatal conductance (g_s), maximal efficiency of PSII (F_v/F_m) were measured using LiCOR model 6400 (Lincoln, Nebraska) equipped with CO₂ control modules and LED light sources. The photosynthetic photon flux density (PPFD) of 1200 μmol m⁻² s⁻¹ was maintained. All measurements were made on fully expanded leaves after 8–9 weeks of growth for vegetative phase and 12–13 weeks of growth for flowering phase. The warm and clear days between 0800 h and 1300 h at saturating light intensities were chosen for measurements. The water pressure deficit was held generally between 1.0 and 1.5 kPa with 25 °C leaf temperature. Flow rate was 20 mmol H₂O mol⁻¹ and Sample cell H₂O was 500 μmol s⁻¹. Water use efficiency (WUE) was calculated by the formula:

$$\text{WUE} = \frac{A_N}{E}$$

Here, A_N = Rate of photosynthesis; E = Transpiration rate.

2.5. Antioxidants, antioxidative enzymes and membrane stability

Reduced ascorbate (ASA) and total ascorbate were determined by following the method of Gillespie and Ainsworth (2007). A standard curve was developed by preparing solutions of ascorbate, 1–50 mmol dissolved in 10% TCA, and analyzing them in the same manner as the extracts. Dehydroascorbate (DHA) was estimated as the difference between total ascorbate and reduced ascorbate.

Total glutathione, and oxidized form of glutathione (GSSG) were estimated according to Griffith (1980). Reduced glutathione (GSH) content was estimated from the difference between total glutathione and GSSG. Standard curves for GSH and GSSG were established over a known concentration range.

The antioxidative enzymes assays were performed by fully matured leaves collected randomly in triplicates from each treatment and control. Based on Superoxide dismutase's (SOD, EC 1.15.1.1) ability to inhibit the reduction of nitro blue tetrazolium (NBT) to form formazan by superoxide the photochemical NBT method (Beyer and Fridovich, 1987) was followed to assay SOD activity. The photoreduction of NBT was measured at 560 nm and one unit of SOD was defined as being equivalent to the volume of extract that caused inhibition of the photoreduction of NBT by 50%.

The method of Rao et al. (1996) was followed for Catalase (CAT, EC 1.11.3.6) activity by measuring the decrease in absorbance at 240 nm due to utilization of H₂O₂. Activity was calculated by using the extinction coefficient 39.4 mM⁻¹cm⁻¹.

The oxidation rate of ascorbic acid measured by the decrease in absorbance at 290 nm was the principle based on which ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured. The method described by Chen and Asada (1989) was followed and activity was calculated by using the extinction coefficient 2.8 mM⁻¹cm⁻¹.

The increase in absorbance (412 nm) when 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) was reduced by glutathione to form 5'-thio-2-nitrobenzoic acid (TNB) (Smith et al., 1988) was measured for glutathione reductase (GR, EC 1.6.4.2) assay. The extinction coefficient 6.2 mM⁻¹cm⁻¹ was used for calculation of enzyme activity.

The 2-thiobarbituric acid (TBA) method (Heath and Packer, 1968)

was adopted for membrane stability assay by estimating the level of lipid peroxidation. The level of lipid peroxidation has direct relation with the amount of malondialdehyde (MDA) formed. MDA was calculated using an extinction coefficient of 155 mM⁻¹cm⁻¹.

2.6. Total soluble sugars, proline and starch

The extraction of total soluble sugars (TSS) and proline from plant tissue was performed by following the method of Cross et al. (2006). The anthrone method was used to estimate TSS. The proline content was assayed through Ninhydrin reagent (Bates et al., 1973). The proline and glucose were used as standards for developing standard curves.

The perchloric acid method described by Whelan (1955) was followed for starch extraction and estimation was performed by Iodine method (Nguyen et al., 2002). The standard curve obtained by using starch over a known concentration range.

2.7. Total leaf protein extraction and two dimensional gel electrophoresis

Total soluble proteins were extracted from leaves of each treatment by grinding 1–1.5 g tissue in Liquid N₂ followed by extraction with buffer [50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 500 mM thiourea and 0.5% 2-mercaptoethanol (BME)]. Protein precipitated overnight at –20 °C by 10 times volume of TCA (trichloroacetic acid)-acetone. After precipitation, three washings of pellet by acetone with 0.07% BME were performed. Second extraction of freeze dried protein pellet after acetone wash by suspending pellet in buffer (0.1 M Tris-HCl, pH 8.0, 50 mM EDTA and 2% BME) then extracted with Tris-buffered phenol followed by overnight ammonium acetate-methanol precipitation at –20 °C. The pellet was dried and then solubilised in solubilisation buffer 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 25 mM DTT (Dithiothreitol) and 0.5% IPG buffer for 2–3 h at room temperature. Subsequently, protein was estimated by Bradford method and stored at –20 °C.

Isoelectric focusing (IEF) was performed in triplicates for each treatment with 7 cm IPG strips, pH 4–7 (Immobilization strip by GE Healthcare) in Ettan IPGphor3 unit (GE Healthcare). The IPG strips were passively rehydrated overnight with 120 μg protein diluted in 8 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% IPG buffer pH 4 to 7, 25 mM DTT, 0.001% bromophenol blue up to a volume of 135 μl. The total proteins was performed in Ettan IPGphor under following conditions: 200 V step for 1 h, 500 V step for 1 h, 4000 V gradient for 2 h, and 6000 V step for 12000 Vhr for a total of 21 kVh. The strips were equilibrated after focusing in equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT for 15 min, and another 15 min in the same buffer replacing DTT by 2.5% (w/v) iodoacetamide. The second dimension was run in Mini-PROTEAN Tetra Cell by BioRad using 7 × 8 cm homogeneous SDS-PAGE gels of 12% T and 5% C. Electrophoresis was performed at constant Voltage 70 V in standard Tris-Glycine running buffer. Gels were stained with 0.5% Brilliant Blue G-250 for 45 min. Destaining was performed in methanol:water:acetic acid (45:45:10 v/v) for 45 min followed by overnight destaining in Milli-Q.

2.8. Image and mass spectrometry analysis

Image analysis was performed using Image Master 2D Platinum 7.0 (GE Healthcare) for protein expression analysis. The relative volume (% volume) was the basis of screening proteins for upregulation and downregulation. The upregulated proteins were with 1.5 fold increase or more in treatment compared to control and downregulated were with 1.5 fold decrease or more in treatment against control.

Mass spectrometry (MS) for the identification of protein spots was performed using 4800 Plus MALDI TOF/TOF Analyzer (ABSCIEX, USA). The protein samples were processed and digested according to Koistinen et al. (2002). The differentially expressed spots were excised

Table 1

Differential expression of *Glycine max* variety JS335 leaf proteins under interactive effect of SA with well watered, water stressed and rehydrated conditions at both stages of development.

JS 335	Vegetative stage ^a				Flowering stage ^a			
	Control ^b	75% ^b	50% ^b	RH ^b	Control ^b	75% ^b	50% ^b	RH ^b
Differentially ^c expressed	38	51	45	57	46	22	51	33
Upregulated ^d	25	45	35	50	26	13	42	30
Downregulated ^e	13	6	10	7	20	9	9	3
Identified ^f	Up↑	33	20	41	36	41	33	15
	Down↓	13	5	8	7	16	4	3

^a Two growth stages of sampling: Vegetative and Flowering.

^b Different conditions: Control- Well watered, 75%–75% RWC (moderate stress), 50%–50%RWC (severe stress), RH-Rehydration, Number represents the proteins with differential abundance in SA treatment under these conditions.

^c Number of differential abundance proteins in SA treatment.

^d Number of proteins with increased abundance in SA treatment.

^e Number of proteins with decreased abundance in SA treatment.

^f Number of proteins identified through mass spectrometry.

and destained by 50 mM ammonium bicarbonate in 50% methanol. Destaining was followed by 3 cycles of alternate dehydration and rehydration in 2:1 v/v solution of acetonitrile and 50 mM ammonium bicarbonate and 25 mM ammonium bicarbonate, respectively. Proteins were trypsin digested by using modified grade Trypsin from Promega in 1:20 ratio of Trypsin to protein at 37 °C overnight. Peptides were eluted thrice with 50% acetonitrile containing 1% Tri-fluoroacetic acid. The recovered peptides were concentrated to a final volume of 3 µl and analysed. The mono isotopic peptide masses obtained were analyzed by the 4000 Series Explorer software version 3.5 (ABI). The protein identification was performed on the basis of mass signals through Mascot software (<http://www.matrixscience.com>) against NCBIprot database. The search criteria in database were as follows: taxonomy, viridiplantae; fixed modification, cysteine carbamidomethylation; variable modification, methionine oxidation; peptide tolerance, ± 1.2 Da, MS/MS tolerance, ± 0.6 Da; peptide charge +1; maximum allowed missed cleavage, 1; instrument type, MALDI-TOF/TOF. The non-probabilistic basis for ranking protein hits and as the sum of the series of peptide scores were protein scores derived from ion scores. The mascot algorithm set the score threshold to achieve $p < 0.05$ based on the size of the database used in the search. False discovery rate (FDR) for protein identification was set to 1%. The protein spots with MOWSE score above threshold level determined by Mascot were considered and proteins with the confidence interval percentage greater than 95% were considered to represent a positive identification.

2.9. Statistical analysis

One way ANOVA (Duncan's test, $p \leq 0.05$) and Linear Mixed Model ANOVA was performed using SPSS 16.0. Protein expression graphs, hierarchical clustering and heat map generation of differentially expressed proteins performed through Multi Experiment Viewer (MeV) software.

2.10. Western blot

Western blot of selected candidate proteins were performed for validating the results of 2DE gel analysis. We have selected ATPase beta (AtpB), GS2, Photosystem II protein (PsbR), Phosphoribulokinase (PRK) and Fructose 1,6 bisphosphate aldolase (FBPase) proteins. Western blot results verified their expression in treated conditions as reported through MS analysis.

2.11. Western blot analysis

Leaf tissues were ground in liquid N₂. Soluble proteins were

extracted from the powdered tissue at 4 °C in 1 ml of buffer (pH = 7.5) containing 50 mM HEPES-KOH, 1 mM EDTA, 5 mM DTT, 10% (v/v) glycerol, 2 mM benzimidazole and 2 mM amino-*n*-caproic acid. After Bradford estimation of protein 7 µg of protein samples were resolved on 10% 1D-PAGE and blotted onto PVDF membrane at 16 V overnight. Tris buffered saline tween (TBST) buffer (pH 7.5) containing 1% bovine serum albumin (BSA) was used to block the blotting membrane for 1 h. The membranes were probed by polyclonal primary antibodies from Agrisera, Sweden against ATPase beta (AtpB, AS05 085), glutamine synthetase (GS2, AS08 296), Photosystem II protein (PsbR, AS05–059), Phosphoribulokinase (PRK, AS09 464) and Fructose 1,6 bisphosphate aldolase (FBPase, AS08 294) proteins at recommended dilution for 2 h in TBST containing 0.25% BSA. PRK was selected for normalization as differential expression of PRK was not observed. HRP conjugated cross-reactive secondary antibody (1:20,000 dilutions) in 0.25% BSA containing TBST buffer was used for probing primary antibody for 2 h. Blot was developed with HRP chromogenic substrate (TMB) for 5–10 min to visualize reactive bands. Image Quant TL 7.0 software was used to perform densitometry analysis for expression pattern.

3. Results and discussion

3.1. Photosynthesis and carbon metabolism

Differential expression profiling in leaf proteins (Tables 1 and S1) showed abundance of photosynthesis related proteins in SA treated control, water stressed and rehydrated plants (Fig. S1–S2, Tables 2 and 3). SA maintained the physiological efficiency under limiting condition indicated by the abundance of RuBisCO activase (RA, spots 39, 47), RuBisCO subunits (spots 12, 28, 33, 36, 56), chl *a/b* binding protein (spot 25), oxygen evolving enhancer protein 1 and 2 (OEE1 & 2, spots 34, 64), Ferredoxin NADP reductase (spots 35, 53, 74), photosynthesis related and PSII stability protein (spots 25, 42, 81) at vegetative stage. The physiological measurements including rate of photosynthesis (A_N), stomatal conductance (g_s) and water use efficiency (WUE) in SA treated water stressed (50 + SA) and rehydrated plants (RH + SA) supported the amelioration of photosynthetic limitation (Figs. 1–2, S3–S4). Hayat et al. (2008) and Askari and Ehsanzadeh (2015) suggested the role of physiological attributes in SA induced tolerance to plants evidenced by proteomics study performed in wheat (Kang et al., 2012; Sharma et al., 2017). Photosynthesis and carbon metabolism, key processes of primary metabolism, have central role in the performance of plants under drought (Lawlor and Tezara, 2009). SA relieved the plants from impaired carbon metabolism under water stress by improved photosynthetic efficiency directing towards carbon assimilation.

Carbon starvation under drought stress is induced by stomatal

Table 2
Representative differentially expressed identified leaf proteins of *Glycine max* variety JS335 under interactive effect of SA with well watered, drought stressed and rehydrated conditions at both stages of development (vegetative and flowering).

Protein	Spot No ^d	Accession No ^e	Score/ empAI ^f	pI/MW ^g	Peptides/% Seq coverage ^h	Fold change ± S.E. ⁱ		50% (V/F)	75% (V/F)	RH (V/F)
						CON (V/F) ^j				
Carbon metabolism^k										
UTP-glucose-1-phosphate uridylyltransferase	1 ^a	XP_003544964.1	254/0.1	5.2/ 56.14	7 (1)/23	1.8 ± 0.2/1 ± 0.1	1 ± 0.02/1 ± 0.02	1 ± 0.04/1 ± 0.06	1 ± 0.01/1 ± 0.2	
Carbonic anhydrase	4/117 ^b	XP_003524544.1	51/ 0.0044	7.59/ 35.8	2 (0)/7	1 ± 0.09/11.89 ± 0.7	12.5 ± 0.1/1 ± 0.04	1 ± 0.1/1.6 ± 0.2	12.5 ± 0.04/11.5 ± 0.09	
	159 ^c	XP_003524544.1	60/11	7.59/ 35.8	2 (0)/8	1 ± 0.02/1.64 ± 0.1	1 ± 0.06/1 ± 0.2	1 ± 0.1/1.96 ± 0.08	1 ± 0.05/1 ± 0.09	
PREDICTED:aldo-1-keto reductase family 4 member C9	6/118 ^b	XP_003520905.2	91/0.09	6.02/ 35.3	4 (0)/12	12.5 ± 1.01/11.5 ± 0.3	1 ± 0.3/1 ± 0.02	1.5 ± 0.1/11.5 ± 0.2	12 ± 0.2/1 ± 0.1	
PREDICTED: fructose biphosphate aldolase 1, chloroplastic	8 ^a	XP_003537836.1	1147/ 2.51	8.24/ 50.6	15 (11)/37	12.68 ± 0.2/1 ± 0.06	1.53 ± 0.06/1 ± 0.2	1.8 ± 0.08/1 ± 0.01	1.5 ± 0.04/1 ± 0.04	
	58/138 ^b	XP_003522841.1	213/11	6.38/ 52.8	14 (10)/40	1 ± 0.03/1 ± 0.02	1.96 ± 0.08/1 ± 0.1	2.3 ± 0.1/2.1 ± 0.3	1 ± 0.2/1 ± 0.05	
Transketolase, chloroplastic	22 ^a	XP_003521870.1	63/11	6.4/82	1 (1)/2	−2.4 ± 0.05/1 ± 0.2	1 ± 0.3/1 ± 0.04	−1.8 ± 0.08 /1 ± 0.1	1 ± 0.4/1 ± 0.06	
	27/126 ^b	XP_003536788.1	61/11	6.12/ 80.69	2 (0)/3	2.89 ± 0.2/1 ± 0.05	2.06 ± 0.2/1 ± 0.03	1 ± 0.04/−1.6 ± 0.2	1 ± 0.2/−1.9 ± 0.2	
Triosephosphate isomerase	43 ^a	NP_001237472.1	370/1.26	5.9/25	5 (4)/20	1 ± 0.06/1 ± 0.02	1.5 ± 0.02/1 ± 0.01	1.98 ± 0.07/1 ± 0.04	1.54 ± 0.05/1 ± 0.07	
Malate dehydrogenase, mitochondrial	45 ^a	KHN10168.1	71/0.18	8.2/36	1 (1)/8	1 ± 0.04/1 ± 0.1	−3.3 ± 0.4/1 ± 0.02	−2.8 ± 0.2/1 ± 0.2	1 ± 0.3/1 ± 0.06	
Phosphoglycerate kinase, cytosolic	46 ^a	XP_003531482.1	260/0.24	7.8/50	5 (2)/26	1 ± 0.2/1 ± 0.07	1 ± 0.05/1 ± 0.02	1.55 ± 0.1/1 ± 0.1	2.2 ± 0.2/1 ± 0.05	
	84/145 ^b	XP_003546820.1	326/0.52	7.8/50	7 (4)/15	1 ± 0.02/1 ± 0.1	1 ± 0.04/1 ± 0.01	1 ± 0.01/1 ± 0.2	1.69 ± 0.2/2.24 ± 0.4	
Phosphoglycolate phosphatase 1B, chloroplastic1like	59 ^a	NP_001276320.1	270/0.53	8.12/ 41.2	4 (3)/10	1 ± 0.09/1 ± 0.2	−1.95 ± 0.1 /1 ± 0.04	1 ± 0.01/1 ± 0.2	1 ± 0.06/1 ± 0.1	
PREDICTED: sedoheptulose1,7- biphosphatase, chloroplastic	62 ^a	XP_003552524.1	663/1.01	60.5/ 42.04	14 (6)/35	1.5 ± 0.1/1 ± 0.07	1.5 ± 0.1/1 ± 0.2	1.9 ± 0.08/1 ± 0.1	1.7 ± 0.2/1 ± 0.06	
Glyceraldehyde-3-phosphate dehydrogenase A subunit	83 ^a	NP_001238484.1	309/11	8.4/ 43.5	8 (1)/25	1 ± 0.05/1 ± 0.03	3.3 ± 0.1/1 ± 0.1	1 ± 0.09/1 ± 0.2	−2.8 ± 0.1 /1 ± 0.02	
	162 ^c	NP_001238484.1	83/11	8.4/ 43.5	3 (0)/7	1 ± 0.02/−2.8 ± 0.5	1 ± 0.06/1 ± 0.1	1 ± 0.02/2.1 ± 0.1	1 ± 0.03/1 ± 0.01	
Photosynthesis^k										
Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic	39/129 ^b 86/147 ^b	NP_001276147.1 NP_001240245.1	12016/11 72/11	6.8/49 6.3/	17 (12)/34 2 (1)/6	1 ± 0.06/2.4 ± 0.04 1 ± 0.1/1 ± 0.09	1 ± 0.2/1 ± 0.01 1 ± 0.05/1 ± 0.02	1.62 ± 0.04/5 ± 0.6 1 ± 0.1/2.2 ± 0.07	1 ± 0.2/1.7 ± 0.2 1.8 ± 0.3/1 ± 0.02	
Ribulose 1,5 biphosphate carboxylase/oxygenase large subunit partial	12 ^a 28 ^a	SBO07506.1 AHF72676.1	434/0.46 977/1.14	6.3/52 6.25/	6 (4)/17 13 (8)/42	1.8 ± 0.5/1 ± 0.1 1.9 ± 0.4/1 ± 0.02	1 ± 0.2/1 ± 0.03 1.95 ± 0.3/1 ± 0.06	1 ± 0.05/1 ± 0.01 4.03 ± 0.1/1 ± 0.2	1 ± 0.2/1 ± 0.06 1 ± 0.3/1 ± 0.1	
	56/137 ^b	SBO07506.1	1097/ 1.84	6.29/ 51.7	13 (11)/36	1 ± 0.1/−2.5 ± 0.3	3.19 ± 0.7/1 ± 0.06	1 ± 0.1/1.74 ± 0.1	1.75 ± 0.2/1 ± 0.2	
Chlorophyll a/b binding protein 3, chloroplastic	25/125 ^b	NP_001241277.1	151/11	5.29/ 27.9	5 (0)/15	2.5 ± 0.2/12 ± 0.3	1.8 ± 0.3/−1.9 ± 0.07	2 ± 0.07/1 ± 0.1	1.98 ± 0.08/1 ± 0.2	
Ribulose1,5 biphosphate carboxylase small subunit rbcS3	33 ^a	AAG24884.1	198/0.28	8.87/ 20.18	4 (1)/21	1 ± 0.04/1 ± 0.2	1 ± 0.05/1 ± 0.03	2.08 ± 0.2/1 ± 0.01	1 ± 0.05/1 ± 0.06	
Oxygen evolving enhancer protein 2, chloroplastic	34 ^a	KHN28540.1	461/0.97	7.68/ 28.59	7 (4)/38	1 ± 0.1/1 ± 0.04	2 ± 0.2/1 ± 0.1	2.3 ± 0.1/1 ± 0.06	1 ± 0.06/1 ± 0.04	
	170 ^c	XP_003519527.1	637/2.35 28.2	7.7/ 28.2	9 (7)/40	1 ± 0.06/1.64 ± 0.04	1 ± 0.04/1 ± 0.1	1 ± 0.2/2.28 ± 0.4	1 ± 0.05/1 ± 0.09	

(continued on next page)

Table 2 (continued)

Protein	Spot No ^d	Accession No ^e	Score/ empAI ^f	pI/MW ^g	Petides/% Seq coverage ^h	Fold change ± S.E. ⁱ CON (V/F)	75% (V/F)	50% (V/F)	RH (V/F)
Ferredoxin/NADP reductase, leaf isozyme, chloroplastic	35 ^a	XP_003547929.1	77/11	8.04/ 40.9	3 (0)/6	1 ± 0.1/1 ± 0.07	1 ± 0.3/1 ± 0.02	2.62 ± 0.4/1 ± 0.1	1 ± 0.2/1 ± 0.1
	53 ^a	XP_003547929.1	867/1.35	6.29/ 51.6	13 (9)/30	1 ± 0.1/1 ± 0.2	5.02 ± 0.2/1 ± 0.07	1 ± 0.02/1 ± 0.05	1 ± 0.3/1 ± 0.1
	93/150 ^b	XP_003547929.1	101/11	8.04/ 40.9	4 (1)/9	1 ± 0.02/11.5 ± 0.9	1 ± 0.1/1 ± 0.05	1 ± 0.05/1 ± 0.02	−2.02 ± 0.1 /1 ± 0.02
Photosystem II stability/assembly factor HCF136, chloroplastic	42 ^a	XP_003540641.1	116/11	6.8/43	4 (0)/12	1.8 ± 0.2/1 ± 0.08	1 ± 0.09/1 ± 0.1	2.48 ± 0.3/1 ± 0.1	1 ± 0.06/1 ± 0.02
PREDICTED: oxygen evolving enhancer protein 1, chloroplastic	63 ^a	XP_003548008.1	772/1.66	6.7/ 35.3	11 (7)/37	1.5 ± 0.1/1 ± 0.04	1.65 ± 0.1/1 ± 0.2	1.9 ± 0.06/1 ± 0.02	1 ± 0.04/1 ± 0.2
	64/139 ^b	XP_004141946.1	385/1.04	6.2/ 35.1	6 (5)/18	1 ± 0.1/1.5 ± 0.04	1.9 ± 0.2/1.6 ± 0.2	1 ± 0.04/3.37 ± 0.1	1.89 ± 0.04/1 ± 0.06
Energy metabolism^k									
ATP synthase subunit alpha, chloroplast	31/127 ^b	Q6L3A1.2	147/11	5.87/ 55.77	4 (1)/11	1.6 ± 0.2/1.5 ± 0.04	1 ± 0.1/1.56 ± 0.1	−3.3 ± 0.7 /1.7 ± 0.03	1.54 ± 0.4/1 ± 0.1
ATP synthase CF1 alpha subunit, chloroplast	57 ^a	YP_538770.1	1145/ 1.79	5.15/ 55.8	20 (11)/33	1 ± 0.1/1 ± 0.03	1.5 ± 0.2/1 ± 0.07	1 ± 0.1/1 ± 0.02	1.5 ± 0.03/1 ± 0.01
	69/142 ^b	AMC30641.1	476/11	5.16/ 55.6	10 (4)/20	1 ± 0.03/1 ± 0.08	2.62 ± 0.6/1 ± 0.1	2.9 ± 0.4/1.5 ± 0.06	1 ± 0.05/1 ± 0.07
Amino acid metabolism^k									
Glutamine synthetase leaf isozyme, chloroplastic	10/119 ^c	XP_003546121.1	508/11	6.73/ 47.94	9 (5)/22	−1.6 ± 0.2/2.6 ± 0.2	2.06 ± 0.09/2.3 ± 0.07	1.5 ± 0.1/2.5 ± 0.5	1.6 ± 0.2/1 ± 0.04
Alanine aminotransferase 2	50/136 ^c	NP_001237567.1	96/11	5.4/ 53.9	3 (1)/4	1.6 ± 0.3/2.3 ± 0.3	1.5 ± 0.2/1.5 ± 0.2	1.6 ± 0.04/1.7 ± 0.1	1 ± 0.04/1.5 ± 0.2
Protein synthesis, assembly and degradation^k									
RuBisCO large subunit binding protein subunit beta, chloroplastic	2/185 ^c	XP_003546771.1	536/0.53	5.7/ 63.15	6 (5)/21	−1.66 ± 0.3/1 ± 0.05	2.06 ± 0.6/1 ± 0.1	1 ± 0.2/2.1 ± 0.1	1 ± 0.09/1.5 ± 0.09
Translation elongation factor EFG	16/121 ^b	CAA50573.1	561/0.32	5.0/78	15 (4)/29	1.5 ± 0.4/1 ± 0.05	1 ± 0.1/1 ± 0.02	1.7 ± 0.1/1.5 ± 0.3	1 ± 0.1/1 ± 0.01
Chaperone protein ClpC, chloroplast	23 ^a	XP_003523172.1	95/11	6.2/103	5 (0)/7	2.07 ± 0.5/1 ± 0.09	2.2 ± 0.01/1 ± 0.06	1.5 ± 0.01/1 ± 0.02	3.9 ± 0.1/1 ± 0.2
Trypsin inhibitor	60 ^a	NP_001237543.1	94/11	6.12/ 18.2	2 (1)/17	1 ± 0.1/1 ± 0.03	2.09 ± 0.2/1 ± 0.1	1 ± 0.04/1 ± 0.01	3.5 ± 0.09/1 ± 0.06
PREDICTED: stromal 70 kDa heat shock1related protein, chloroplastic	65/140 ^b	XP_003548186.1	1044/ 0.84	5.2/ 73.9	15 (9)/22	1 ± 0.01/1.83 ± 0.02	1.57 ± 0.3/2.9 ± 0.2	2.06 ± 0.1/1 ± 0.04	1 ± 0.09/1 ± 0.06
PREDICTED: elongation factor Tu, chloroplastic1like	68 ^a	XP_003523105.1	137/11	6.2/ 52.3	4 (0)/12	1 ± 0.02/1 ± 0.05	2.06 ± 0.3/1 ± 0.1	2.4 ± 0.06/1 ± 0.2	1 ± 0.1/1 ± 0.01
Metalloendoproteinase 1 precursor	154 ^c	NP_001235535.1	570/1.11	6.3/ 34.1	9 (5)/31	1 ± 0.1/1.52 ± 0.1	1 ± 0.02/1.97 ± 0.06	1 ± 0.01/1.6 ± 0.09	1 ± 0.05/1 ± 0.03
Peptidyl prolyl cis/trans isomerase, chloroplastic	168 ^c	XP_003536011.1	185/0.45	8.5/ 29.3	4 (2)/16	1 ± 0.01/−2.3 ± 0.2	1 ± 0.1/1 ± 0.07	1 ± 0.05/1.9 ± 0.05	1 ± 0.2/1 ± 0.05
Defense^k									
Ascorbate peroxidase 1, cytosolic	26 ^a	NP_001237785.1	523/1.59	5.51/ 27.75	10 (5)/42	1.6 ± 0.1/1 ± 0.05	1.54 ± 0.1/1 ± 0.02	1.8 ± 0.3/1 ± 0.08	1.7 ± 0.2/1 ± 0.2
2-Cys peroxiredoxin BAS1, chloroplast	169 ^c	XP_003519127.1	203/0.42	5.97/ 28.7	4 (2)/18	1 ± 0.05/1 ± 0.02	1 ± 0.07/−1.84 ± 0.1	1 ± 0.03/1 ± 0.01	1 ± 0.04/1 ± 0.1
Ascorbate peroxidase 2	177 ^c	AAB01221.1	226/0.2	5.7/27	6 (1)/30	1 ± 0.06/1 ± 0.01	1 ± 0.07/2.4 ± 0.1	1 ± 0.02/1.6 ± 0.1	1 ± 0.04/1.8 ± 0.07
Redox signaling^k									
Thioredoxin-like protein CDSF32, chloroplast	21 ^a	XP_003545009.1	71/11	6.3/33	3 (0)/15	1.5 ± 0.06/1 ± 0.08	1.57 ± 0.06/1 ± 0.01	1.6 ± 0.2/1 ± 0.04	4.2 ± 0.2/1 ± 0.2
PREDICTED: thioredoxin-like protein CDSF32, chloroplastic	75/144 ^b	XP_003545009.1	58/0.17	6.32/ 33.3	2 (1)/8	1 ± 0.03/1 ± 0.1	3.5 ± 0.3/−2.4 ± 0.06	1 ± 0.02/−1.8 ± 0.09	1 ± 0.01/1 ± 0.1
Secondary metabolism^k									
Isoflavone reductase homolog 2	41/130 ^b	NP_001237637.1	135/0.16	5.6/34	3 (1)/10	1 ± 0.03/1 ± 0.1	1 ± 0.06/1 ± 0.05	1.55 ± 0.2/1 ± 0.06	1 ± 0.06/1.5 ± 0.1
Cytoskeleton^k									

(continued on next page)

Table 2 (continued)

Protein	Spot No ^d	Accession No ^e	Score/ emPAI ^f	pI/MW ^g	Peptides/% Seq coverage ^h	Fold change ± S.E. ⁱ		75% (V/F)	50% (V/F)	RH (V/F)
						CON (V/F)				
Actin	24 ^a	XP_003519037.1	367/0.44	5.3/ 41.8	6 (3)/17	1.76 ± 0.3/1 ± 0.02	1.69 ± 0.4/1 ± 0.01	3.4 ± 0.08/1 ± 0.1	1.9 ± 0.05/1 ± 0.09	

^a Spot No at Vegetative stage.
^b Spot No at Vegetative stage/Spot No at Flowering stage.
^c Spot No at Flowering stage.
^d Spot numbers correspond with 2-D gel as shown in Figure.
^e Accession number in NCBI database.
^f Protein score was based on combined MS and MS/MS spectra.
^g Protein pI/MW (kDa): pI protein/molecular mass of protein.
^h Peptides/% Seq coverage: Number of peptides matched with the protein/percentage of protein sequence covered.
ⁱ Fold changes were from three biologically independent experiments of 2-DE, compared with the control ± Error of fold change: CON, Control + SA (CON + SA)/Control (C); 75%, 75 + SA (75 + SA)/75% (75%); 50%, 50 + SA (50 + SA)/50% (50%); RH, RH + SA (RH + SA)/RH (RH).
^j V/F = Fold change at Vegetative stage/Fold change at Flowering stage.
^k Functional Category. Different spot ids for same protein at two stages as matching was performed between SA treated and non-treated samples for both stages.

Table 3
Functional characterization of identified differentially expressed proteins of *Glycine max* variety JS335 under SA treatment at Vegetative stage and Flowering stage.

Functional Category	Percentage (%) of total identified proteins	
	Vegetative Stage	Flowering Stage
Photosynthesis	30	39
Carbon metabolism	26	21
Protein Synthesis, Assembly and Degradation	17	18
Energy Metabolism	12	7
Amino Acid Metabolism	2	3
Defense	1	4
Redox Signaling	4	2
Cell Signaling	1	2
Secondary Metabolism	2	2
Cytoskeleton	1	–
Unknown	4	2

closure and reduced photosynthesis impairing carbon metabolism (Chaves et al., 2009; Gilbert et al., 2011). Decreased g_s under mild stress (75 + SA) have protective effects against stress, improving water use efficiency (WUE, Fig. S3a) without affecting rate of photosynthesis (Chaves et al., 2009). Abundance of carbonic anhydrase (CA, spot 76) increased the CO_2 concentration around RuBisCO increasing its carboxylation efficiency in 75 + SA plants influencing A_N (Gillon and Yakir, 2000). The leaves enter senescence coincident with flowering which may be linked to resource allocation or organ turnover (Thomas, 2013). During flowering stage, synergistic effects of drought and salicylic acid induce senescence in 50 + SA plants observed through decrease in chlorophyll content and photosynthetic rate (Fig. 1b & S5b). The increased expression of RuBisCO (spots 137), RA (spots 120, 129, 160, 163, 173, 178, 184), CA (spots 117, 159), OEE1 & 2 (spots 139, 165, 170) and PSII related proteins (spots 149, 170, 165) under SA treatment (Tables 2 and S1-S2, Fig. S1-S2) protected the decline in rate of photosynthesis due to senescence. In agreement with Hao et al. (2015) and Sharma et al. (2017), water stressed plants after rehydration under control and SA treatment (RH and RH + SA) showed complete recovery of physiological functions corroborated with proteomics (Figs. 1–2 & S3-S4; Tables 2 and S1-S2).

Western blot results corroborated the proteomics result as similar expression observed in PSII proteins (Fig. S6-S7). The reducing power of NADPH, regenerated through Ferredoxin NADP reductase, was used in carbon fixation, nitrogen metabolism, and lipid and chlorophyll biosynthesis and redox regulation under stress (Rumeau et al., 2007). Carbon gain through improved photosynthetic efficiency under SA maintained plant vigor under stressful environment. SA relieved plants from carbon impairment observed through increased starch and total soluble sugar (TSS) accumulation in SA treated water stressed (50 + SA, 75 + SA) and rehydrated (RH + SA) plants (Fig. 3 & S8). The decreased abundance of TK (spots 27, 48, 72, 94) in SA treated plants relieved inhibition from starch biosynthesis (Henkes et al., 2001). Abundance of Triose phosphate isomerase (TPI, spot 43) and Fructose biphosphate aldolase, chloroplastic (FBPase, spots 8, 9, 58, 99, 110), key enzymes in soluble sugar synthesis, increased the soluble sugar in SA treated plants for osmoregulation under water stress (Ito et al., 2003). SA induced abundance of FBPase and TPI together with aldo keto reductase (spot 6), enzyme involved in sorbitol synthesis, caused predominant increase in sugars for maintaining osmotic balance in 50 + SA plants as supported by Tari et al. (2010). Western blot results substantiated the abundance of FBPase under SA treatment (Fig. S6-S7).

FBPase together with sedoheptulose 1,7 biphosphatase (SBPase; spot 62) play key role in RuBP regeneration maintaining high rate of photosynthesis under SA treatment. Glycolytic enzymes

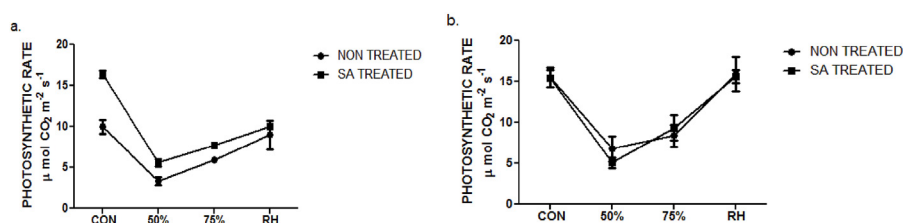


Fig. 1. Rate of photosynthesis (A_N) in *Glycine max* variety JS335 under the interaction of SA with water stress (75% and 50%) and rehydration at a. Vegetative stage and b. Flowering stage. Mixed model ANOVA analyzed level of significance ($p \leq 0.05$) for main effects and interaction: Treatment (TRT) $p < 0.001$; Time (TM) $p < 0.001$ and TRT*TM $p < 0.001$.

Phosphoglycerate kinase (PGK, spots 46, 77, 84) and Glyceraldehyde-3-phosphate dehydrogenase (GAPD, spot 83) abundance in 50 + SA and 75 + SA plants directed towards carbon assimilation (Kang et al., 2012; Sharma et al., 2017). The decreased abundance of mitochondrial malate dehydrogenase (mMDH, spot 45) and Phosphoglycolate phosphatase (PGPase, spot 59) resulted in energy shift from photorespiration to carbon assimilation (Tomaz et al., 2010). The rate of respiration restored in rehydrated plants under SA treatment was evidenced by abundance of mMDH (spot 82) while decrease in peroxisomal glycolate oxidase (spot 100) combated the excess production of H_2O_2 (ROS) through photorespiration as observed in rewatered tobacco plants (Galle et al., 2010). In agreement with Shakirova (2007), dry matter accumulation in shoots of SA treated soybean plants substantiated carbon gain ameliorating drought limitations (Fig. S8-S9). Carbon assimilation from flowering stage showed shift from source to sink for yield stability. Abundance of TK (spots 126, 167) and TPI (spot) explained the shift towards sugars like sucrose that can be translocated to sink (Fig. 3 & S8).

3.2. Energy and amino acid metabolism

The abundance of different ATP synthase and their subunits (spots 7, 32, 57, 66, 69, 70, 127, 142, 179) under SA generated ATP from PSII driven proton gradient at both stages in water stressed and rehydrated plants. SA facilitated the plants for fulfilling the energy requirement to cope up with stress and maintaining growth and development (Kang et al., 2012). Nitrogen assimilation positively influenced under SA treatment providing building blocks for proteins showing metabolic and protective functions against stress (Sharma et al., 2017). In agreement, Amino transferase (AlaAT, spot 50), a carbon–nitrogen shuttle, plastidic glutamine synthetase (GS, spot 10) abundance in SA treated soybean plants depicted protective role of SA against drought stress (Table 2 & Table S1-S2). Gln can serve as a reservoir of nitrogen and is the major form of reduced nitrogen in the phloem sap during nitrogen remobilization (Shrawat et al., 2008). The increased expression of GS (spot 119) and AlaAT (spot 136) during flowering stage (Fig. 6b, Tables 2 and S1-S2) under SA influenced the nitrogen remobilization in senescing organs by the synthesis of Gln thereby increasing NUE and ultimately the yield as reported by others (Martin et al., 2006; Good et al., 2007; Shrawat et al., 2008).

3.3. Protein synthesis, assembly and degradation

Abundance of elongation factor G and Tu (EF-G and EF-Tu, spots 16, 68, 79, 87) under SA provided proteins for metabolic functions improving drought tolerance. Impaired protein synthesis, protein misfolding and degradation are some of the major detrimental effects of

drought stress in plants. The protein misfolding due to stress corrected by the SA induced abundance of different heat shock proteins (Hsps, spots 65, 71, 90, 91), chaperons ClpC (spots 23, 73) and RuBisCO large subunit binding protein (RBP, spots 2, 38, 185) in 50 + SA, 75 + SA and RH + SA. Protease inhibitor protein like trypsin inhibitor (TI, spot 60) in 75 + SA and RH + SA inhibited the function of proteases leading to stability of the respective proteins otherwise degraded by stress (Srinivasan et al., 2009). ATP dependent zinc metalloprotease FTS2 (spot 109) and Cysteine proteinase (CP, spot 114) have been reported to maintain protein quality and proteome homeostasis through sequestering potentially harmful misfolded proteins (Vaseva et al., 2012; Sedaghatmehr et al., 2016) explaining its increase in re-watered plants (RH + SA) by SA treatment. The protein turnover and homeostasis was maintained to provide proteins (proteases, transporters etc) involved in nitrogen remobilization during source to sink transitions (flowering stage). In agreement, SA treatment facilitated the soybean plants to maintain the homeostasis by proteins involved in synthesis (EF-Tu & G, spots 121, 148, 175), assembly (Hsps and Protein P21, spots 140, 181) and degradation (proteases, spots 153, 154, 168; Table S1-S2). The proteases are reported to be important for developing N pool in source leaves for transition/translocation to sink (Donnison et al., 2007; Liu et al., 2008).

3.4. Defense responses

Metabolic energy regenerated through improved photosynthetic efficiency under SA treatment was utilized in carbon, nitrogen and protein metabolism generating defense responses against drought. SA induced abundance of AlaAT and GS increased the nitrogen assimilates (glutamine, Gln and glutamate, Glu) directed towards proline accumulation for osmoregulation and redox buffering throughout soybean development (Table 2; Fig. 4 & S6-S7; Shrawat et al., 2008; Verslues and Sharma, 2010). SA maintained the osmotic balance under drought generated osmotic stress through accumulation of TSS as mentioned earlier. SA treatment under water stress in plants had earlier reported to increase the levels of different osmolytes for stress tolerance (Farooq et al., 2009; Sharma et al., 2017). Pinheiro and Chaves (2010) demonstrated the interplay between ROS scavenging system and soluble sugars under drought. Soluble sugars signal ROS scavenging system inducing different antioxidative enzymes and antioxidants against oxidative stress. Chen et al. (2016a) reported that SA treatment leads to enhanced antioxidant capacity of plants by increasing the enzymes activity and antioxidants level. Increased activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) observed in SA treated soybean plants under control and stressed conditions at vegetative stage (Fig. 5). During flowering stage, senescence induced ROS production scavenged by increased

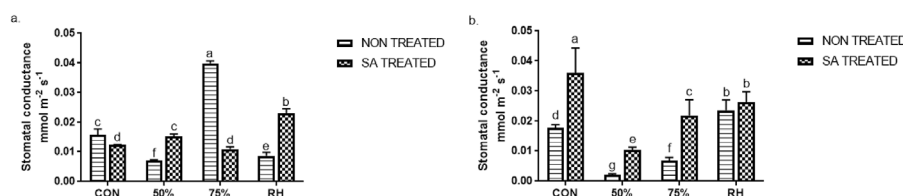
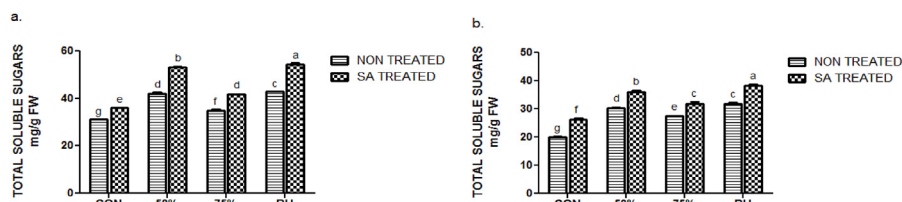
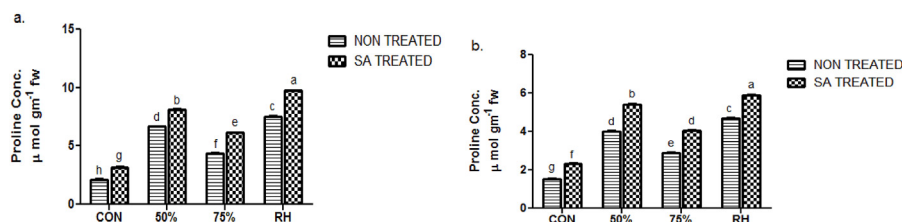


Fig. 2. Stomatal conductance (g_s) in *Glycine max* variety JS335 under the interaction of SA with water stress (75% and 50%) and rehydration at a. Vegetative stage and b. Flowering stage. Bar represents mean \pm standard deviation ($n = 3$), letters (abc) represents level of significance through one way ANOVA post hoc Duncan's test ($p \leq 0.05$). Mixed model ANOVA analyzed level of significance ($p \leq 0.05$) for main effects

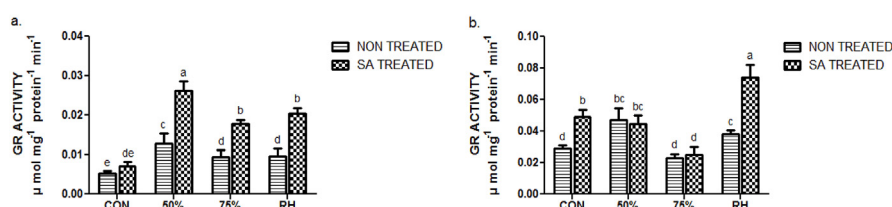
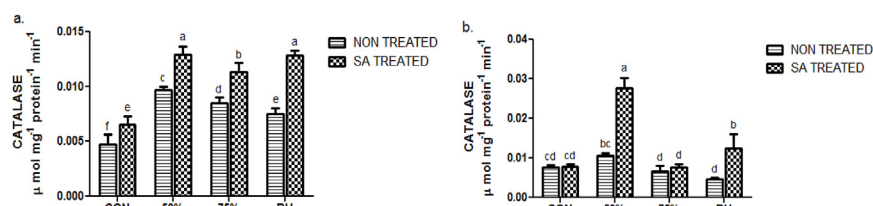
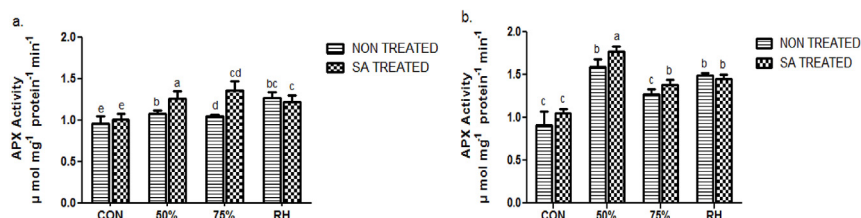
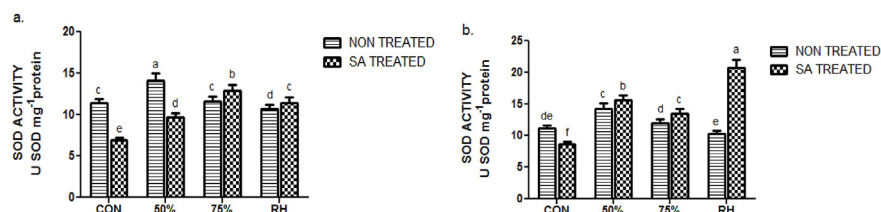
and interaction: Treatment (TRT) $p < 0.001$; Time (TM) $p = 0.088$ and TRT*TM $p < 0.001$.



$p < 0.001$ and $TRT \times TM$ $p < 0.001$.



$p < 0.001$.

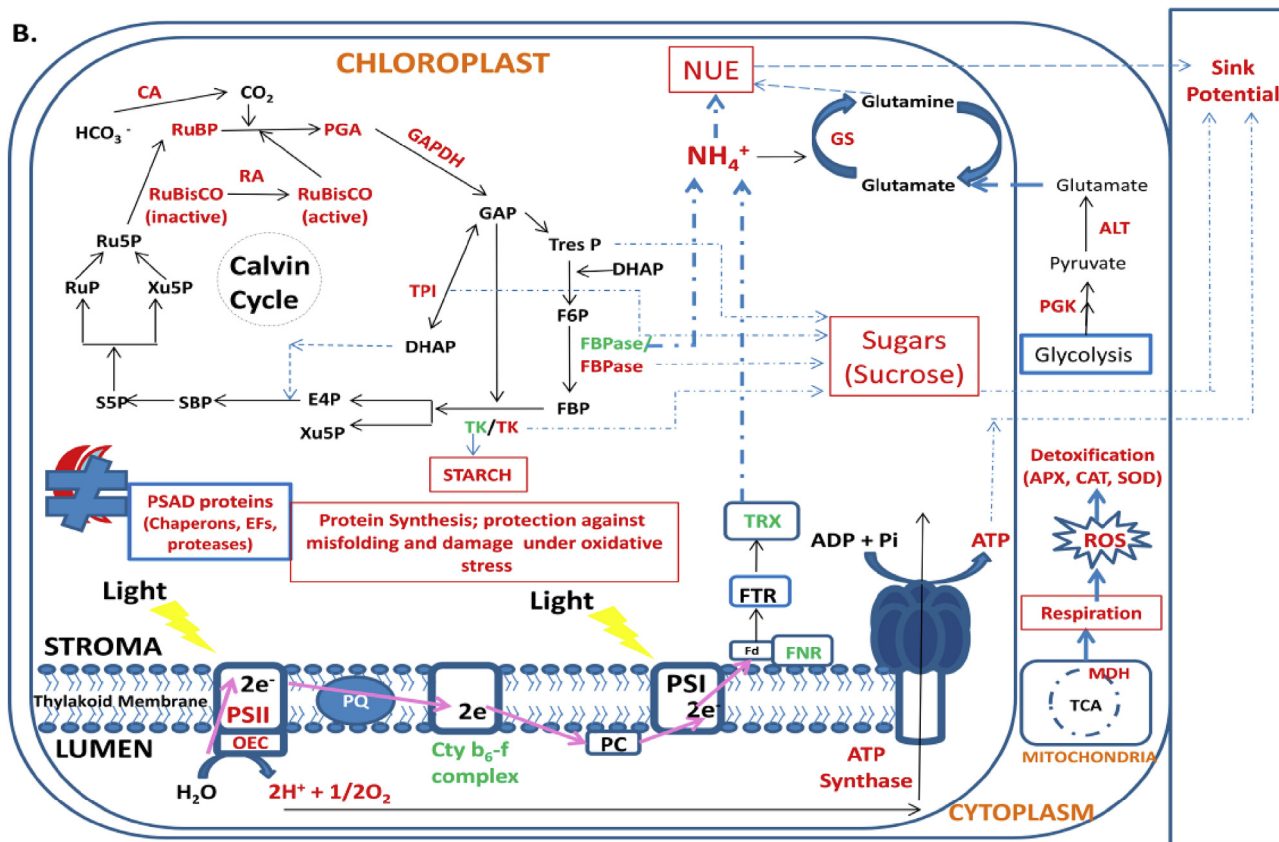
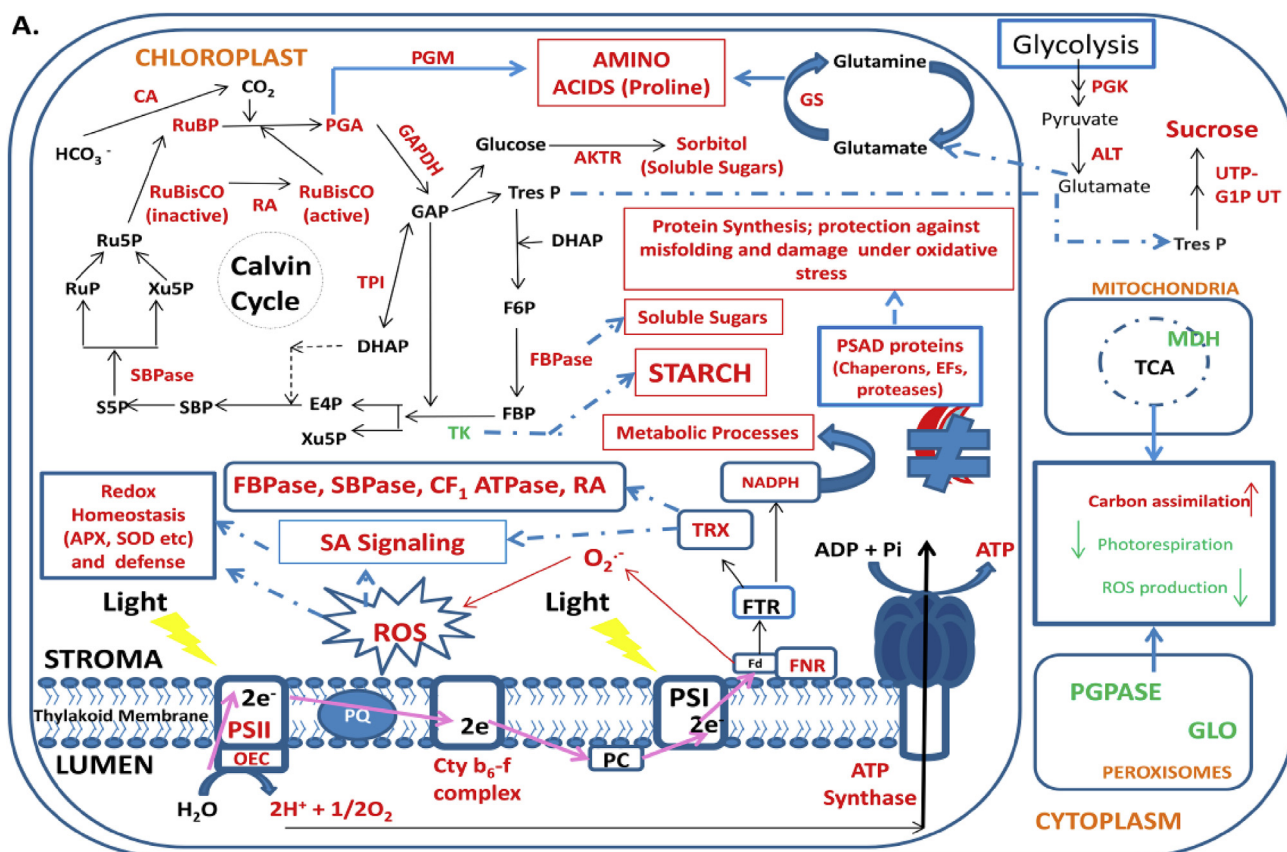


activity of APX, CAT and SOD in SA treated plants under water stress (Fig. 5b, Rogers and Sergi Munné-Bosch, 2016). Differential protein profiling demonstrated the abundance of APX (spots 26, 177) under SA treatment corroborating with the biochemical analysis (Tables 2 and S1-S2). Antioxidant ascorbate and glutathione level showed predominant increase in stressed and rehydrated plants under SA treatment providing an efficient ROS scavenging system under water deficit throughout the soybean development (Figs. S10-S11). Saruhan et al. (2012) findings on SA induced alleviating effect on drought stress by

Fig. 3. Total soluble sugars content in *Glycine max* variety JS335 under interaction of SA with water stress and rehydration at a. Vegetative and b. Flowering stage. Bar represents mean \pm standard deviation ($n = 3$), letters (abc) represents level of significance through one way ANOVA post hoc Duncan's test ($p \leq 0.05$). Mixed model ANOVA analyzed level of significance ($p \leq 0.05$) for main effects and interaction: Treatment (TRT) $p < 0.001$; Time (TM)

Fig. 4. Proline content in *Glycine max* variety JS335 under interaction of SA with water stress and rehydration at a. Vegetative and b. Flowering stage. Bar represents mean \pm standard deviation ($n = 3$), letters (abc) represents level of significance through one way ANOVA post hoc Duncan's test ($p \leq 0.05$). Mixed model ANOVA analyzed level of significance ($p \leq 0.05$) for main effects and interaction: Treatment (TRT) $p < 0.001$; Time (TM) $p < 0.001$ and $TRT \times TM$

Fig. 5. Activity of antioxidant enzyme Superoxide dismutase (SOD), Ascorbate peroxidase (APX), Catalase (CAT) and Glutathione reductase (GR) under the interactive effect of SA with water stress (75% and 50%) and rehydration at a. Vegetative and b. Flowering stage. Bar represents mean \pm standard deviation ($n = 3$), letters (abc) represents level of significance through one way ANOVA post hoc Duncan's test ($p \leq 0.05$). Mixed model ANOVA analyzed level of significance ($p \leq 0.05$) for main effects and interaction: Treatment (TRT) $p < 0.001$ (SOD, APX, CAT and GR); Time (TM) $p < 0.001$ (SOD and GR), $p = 0.939$ (APX), $p = 0.001$ (CAT) and $TRT \times TM$ $p < 0.001$ (SOD, CAT and GR), $p = 1$ (APX).



(caption on next page)

Fig. 6. Diagrammatic representation showing effects of SA treatment on soybean plants under water stress and rehydration at a. Vegetative stage and b. Flowering stage. Red colour represents positive regulation and green represents negative regulation. Abbreviations: CA: Carbonic anhydrase; PGA: 3-Phosphoglycerate; RuBisCO: Ribulose 1,6 biphosphate Carboxylase/Oxygenase; RA: RuBisCO Activase; GAP: Glyceraldehyde 3-phosphate; GADPH: Glyceraldehyde 3 phosphate dehydrogenase; PGM: Phosphoglycerate mutase; PGPase: Phosphoglycolate Phosphatase; DHAP: Dihydroxy acetone phosphate; TK: Transketolase; TPI: Triose phosphate isomerase; Tres P: Triose phosphate; MDH: Malate Dehydrogenase; FBP: Fructose 1,6 biphosphate; F6P: Fructose 6 phosphate; UTP-G1P UT: UTP Glucose 1 phosphate Uridyl transferase; E4P: Erythrose 4-phosphate; FBPase: Fructose biphosphate aldolase; Xu5P: Xylulose 5-phosphate; Ru5P: Ribulose 5 phosphate; SBP: Sedoheptulose 1,7 biphosphate; S5P: Sedoheptulose 7 phosphate; GS: Glutamine synthetase; ALT: Alanine aminotransferase; AKTR: Aldo keto reductase; PGK: Phosphoglycerate kinase; GLO: Glycolate oxidase; OEC: Oxygen evolving complex; FNR: Fd-NADP Reductase; Trx: Thioredoxin; PSAD: Protein synthesis, assembly and degradation; EFs: Elongation factors; ROS: Reactive oxygen species; NUE: Nitrogen Use Efficiency. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

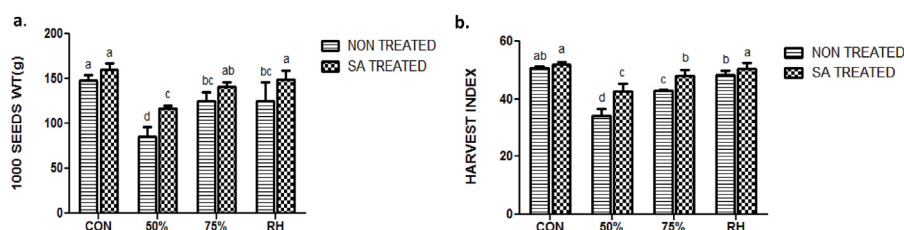


Fig. 7. Yield parameters a. Thousand seed weight; b. Harvest index (HI) under interaction of drought and rehydration with SA in *Glycine max* variety JS335. Bar represents mean \pm standard deviation ($n = 3$), letters (abc) represents level of significance through one way ANOVA post hoc Duncan's test ($p \leq 0.05$).

Trx CDSP32 (spot 144) and Cys peroxiredoxin BAS1 (Cys-Prx, spot 169; Tables 2 and S1-S2) explained the increase in MDA concentration at flowering stage (Fig. S12b).

3.5. Cytoskeleton and secondary metabolism

2-methylene furan-3-one (spot 29) in 50 + SA and RH + SA plants demonstrated the role of SA in enhancing furan fatty acids (F-acids) serving as radical scavengers defending plants against oxidative stress (Mawlong et al., 2016). Isoflavone reductase (IFR, spots 41, 130), involved in isoflavonoid biosynthesis, abundance in 50 + SA plants also provided tolerance to soybean against ROS produced under drought stress (Kim et al., 2010; Chen et al., 2016b). Actin protein overexpression is among one of the key trait in conferring drought tolerance (Śniegowska-Świerk et al., 2015). In agreement, SA induced abundance of actin (spots 24, 124) maintained the cytoskeleton and positioning of organelles like chloroplast under stress condition restoring their functions for plant survival.

3.6. Interplay between redox signaling and SA signaling

SA induced abundance of Trx CDSP32 (spots spots 20, 21, 75) in water stressed soybean plants depicted the interplay between photosynthesis, redox signaling and SA signaling triggering defense responses against drought (Tables 2 and S1-S2; Fig. 6a). Reducing power, generated from PSI mediated redox signaling through redox sensors like Trxs and Prxs in chloroplasts, helped in triggering defense responses (Dietz et al., 2006; Nikkanen et al., 2016). ROS accumulation under drought stress triggers redox signaling through redox sensor or signaling molecule Prxs in chloroplast (Dietz et al., 2006; Liebthal et al., 2018). Redox signaling in chloroplast interplay with SA signaling under stress triggering different defense responses (Foyer et al., 2014; Xia et al., 2015; Dietz et al., 2016). Sharma et al. (2017) reported the interplay between redox signaling and SA signaling in SA induced drought tolerance in wheat.

3.7. Yield

Improved yield parameters like seed starch, seed weight per plant, seed number per plant, thousand seed weight and harvest index demonstrated the yield stability in SA treatment under water limiting environment (Fig. 7 & S13-S14). Fariduddin et al. (2003) reported positive influence of SA treatment on yield as observed in 50 + SA and 75 + SA plants. Two seed quality parameters, seed weight per plant and seed number per plant, were significantly increased in control (C)

soybean plants (Fig. S13). Plants rehydrated after severe stress showed significant increase in seed starch, thousand seed weight and harvest index on SA treatment (Fig. 7 & S14). The thousand seed weight increase was more predominant in rehydrated plants then in 50 + SA and 75 + SA (Fig. 7a). The difference in TSW was due to decrease in seed number in rehydrated plants and small increase in 50 + SA as compared to 75 + SA (Fig. 7a & S13b). The results were supported by the finding of Sadras (2007) who reported a negative correlation between the number of seeds produced and the thousand seed weight. The ameliorating role of SA on yield limited by water stress studied through different yield parameters was supported by Sharafizad et al. (2012) and Sharma et al. (2017). Nitrogen use efficiency (NUE) is essential during anthesis for sustainable yield under drought stress. SA pre-treated soybean plants showed improved sink potential even under yield limiting water stress. The downregulation of acid phosphatase haloacid dehalogenase like hydrolase (spot 164) in CON + SA and RH + SA was a requirement for pod storage protein to function as storage protein in soybean (Table S1-S2; Leelapon et al., 2004). The nitrogen remobilization to sink caused downregulation of different proteins under SA in soybean during flowering for N allocation e.g., RuBisCO subunits (spots 136, 137), photosynthesis related proteins (spots 93, 125, 155, 174), RBP (spot 128), Trx CDSP32 (spot 144), Cys-Prxs (spot 169), Hsps (spot 143) and Peptidyl prolyl isomerase (PPI, spot 168) stabilizing the yield (Table S1-S2; Sharma et al., 2017). The leaves behave as a source for N through protein degradation for developing sink organs like seed during flowering, further induced by SA in control and stressed conditions. The improved sink strength under SA treatment in soybean plants attributed towards the increase in yield parameters thereby positively influencing the soybean productivity.

4. Conclusion

Salicylic acid induced alterations and modulations helped the plants to attain energy and developmental equilibrium to cope up with the stress. SA modulated the physiological and metabolic processes towards the survival and acclimation of soybean plants to water stress. The reducing energy produced through improved photosynthesis directed towards carbon, nitrogen and protein metabolism to maintain plant growth and vigor under drought. SA induced defensive mechanisms driven through metabolic processes involved in osmoregulation, redox homeostasis and protein homeostasis conferred drought tolerance. Redox signaling in chloroplast under drought generated oxidative stress interplay with SA signaling triggered different defense responses under water stress. The yield limitations under water stress was abrogated by improved sink strength through different processes occurring during

flowering for increased nitrogen use efficiency and assimilate partitioning/allocation to developing sink. The series of parallel processes undergoing in development of soybean plants under interaction of SA and drought facilitated the plants with improved tolerance ultimately acquiring yield stability. The emerging paradigm is plant tolerant response to drought under SA occurring via series of rapid and parallel developing physiological, cellular and molecular events. The future studies should focus on ameliorating role of SA from drought with the major goal of yield stability in stressful environment.

Authors' contribution

VP, AA and MS designed the experiment. MS, SKG and VKM did yield and physiological work. BM did the enzymatic work. FD, MS and SKG did proteomic work. MS and VP analysed the data. MS, AA and VP wrote the paper. All the authors approved the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.plaphy.2018.08.001>.

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