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Salicylic acid mediated growth, physiological and proteomic responses in two wheat varieties under drought stress

Marisha Sharma [a,b], Sunil K. Gupta [a], Baisakhi Majumder [a], Vivek K. Maurya [a], Farah Deeba [a], Afroz Alam [b], Vivek Pandey [a,*]

[a] Plant Ecology and Environmental Science Division, CSIR-National Botanical Research Institute, Lucknow 226001, India
[b] Department of Bioscience and Biotechnology, Banasthali University, P.O. Banasthali Vidyapith, 304022, Rajasthan, India

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ABSTRACT

Salicylic acid (SA) induced drought tolerance can be a key trait for increasing and stabilizing wheat production. These SA induced traits were studied in two *Triticum aestivum* L. varieties; drought tolerant, Kundan and drought sensitive, Lok1 under two different water deficit regimes: and rehydration at vegetative and flowering stages. SA alleviated the negative effects of water stress on photosynthesis more in Kundan. SA induced defense responses against drought by increasing antioxidative enzymes and osmolytes (proline and total soluble sugars). Differential proteomics revealed major role of carbon metabolism and signal transduction in enhancing drought tolerance in Kundan which was shifted towards defense, energy production and protection in Lok1. Thioredoxins played important role between SA and redox signaling in activating defense responses. SA showed substantial impact on physiology and carbon assimilation in tolerant variety for better growth under drought. Lok1 exhibited SA induced drought tolerance through enhanced defense system and energy metabolism. Plants after rehydration showed complete recovery of physiological functions under SA treatment. SA mediated constitutive defense against water stress did not compromise yield. These results suggest that exogenously applied SA under drought stress confer growth promoting and stress priming effects on wheat plants thus alleviating yield limitation.

Biological significance: Studies have shown morphological, physiological and biochemical aspects associated with the SA mediated drought tolerance in wheat while understanding of molecular mechanism is limited. Herein, proteomics approach has identified significantly changed proteins and their potential relevance to SA mediated drought stress responses in drought tolerant and sensitive wheat varieties. SA regulates wide range of processes such as photosynthesis, carbon assimilation, protein metabolism, amino acid and energy metabolism, redox homeostasis and signal transduction under drought. Proteome response to SA during vegetative and reproductive growth gave an insight on mechanism related water stress acclimation for growth and development to attain potential yield under drought. The knowledge gained can be potentially applied to provide fundamental basis for new strategies aiming towards improved crop drought tolerance and productivity.

1. Introduction

Drought is an insidious hazard of nature causing extensive damage to crops, resulting in loss of yield. Wheat is one of the world's most important food crop providing 20% of all calories and 20% of all protein in developing and developed countries [1]. Prevalent drought conditions could decline its yield between 23 to 27% in world's leading wheat belts by 2050 [2]. Extensive research has been done in combating the damage caused by water deficit on wheat [3,4,5,6,7] The role of salicylic acid (SA), a multifunctional plant hormone, in ameliorating damage caused to plants due to water stress has been substantiated. The role of SA in regulation of drought supported by the increase in endogenous level and the induction of SA-inducible genes *PR1* and *PR2* by drought stress [8]. The *Arabidopsis* mutants *adr1*, *myb96-1d*, *siz1*, *acd6*, and *cpr5* reported to accumulate endogenous SA and exhibited SA-dependent drought tolerance [8,9,10]. SA-accumulating mutants (*cpr5* and *acd6*) of *Arabidopsis thaliana* exhibited improved drought tolerance due to stomatal closure by SA-mediated induced expression of PR genes (PR1, PR2, and PR5) [11]. Pal et al. [12] reported the correlation between ortho-hydroxy-cinnamic (oHCA) with SA biosynthesis and its crucial role in drought tolerance in *Oryza sativa*. SA-altering Arabidopsis mutants, *snc1* (with constitutively high levels of SA), displayed alleviation of PEG-induced growth inhibition, leaf water loss, and photosynthesis-related impairment and maintained the osmotic potential by increased levels of proline [13]. Martin et al., [14] reported the positive influence of SA accumulation on stomatal opening, photorespiration and antioxidant defenses in oats. SA increased carbon metabolism, antioxidant

* Corresponding author.
E-mail address: v.pandey@nbri.res.in (V. Pandey).

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system, membrane stability, osmoprotection and photosynthetic pigments in wheat [15], barley [16,17], tomato [18], rice [19], chickpea [20] and maize [21]. Rajjou et al. [22] reported the positive influence of SA pre-treatment on content of antioxidant enzymes for establishing early defense mechanisms, and the mobilization of seed storage proteins through proteomics in *Arabidopsis*. SA treated water stressed wheat plants had higher level of sugars, protein and mineral contents [23] and solutes (organic and inorganic) [24]. SA effectively increased the net CO_2 assimilation rate with an increase in shoot dry matter which helped *Torreya grandis* to acclimate to drought stress [25]. The enhanced drought tolerance could be mainly due to the increased content of amino acids and carbohydrates involved in osmotic adjustment (OA) and energy metabolism [26]. The mechanisms underlying drought tolerance induced by exogenous SA at the molecular level have been studied. Kang et al. [27] revealed 35 key proteins involved in the SA-responsive protein interaction network suggesting that these proteins are critical for SA-induced tolerance. SA-induced growth and drought tolerance in wheat seedlings involved proteins associated with signal transduction, stress defense, photosynthesis, carbohydrate metabolism, protein metabolism, and energy production. SA alleviates the detrimental effects of drought stress on wheat seedling growth by influencing the ascorbic acid-glutathione (ASA-GSH) cycle as studied through the transcription of eight genes related to the cycle [28]. The genes encoding for chaperone, HSPs, antioxidants and secondary metabolite biosynthesis, such as SAD, CAD, and Cytochrome P450 (CYP) responded to SA treatment and correlated with signaling pathway in plants under drought stress condition [29]. The positive correlation between transcription regulator gene *AtZAT6* overexpression and expression levels of SA-related genes was observed in providing drought tolerance to plants [30]. Although, SA induced changes in drought stressed wheat plants have been studied, proteomic changes at vegetative and flowering stages have seldom been studied.

The present study assesses the impacts of SA on drought stressed wheat (*Triticum aestivum* L.) varieties (Kundan and Lok1) at the morphological, physiological, biochemical and proteome level to further explore the molecular mechanism(s) underlying SA-induced drought tolerance. Kundan and Lok1 are high yielding varieties sown in Indo-gangetic plains [31,32]. They show contrasting drought tolerance, Kundan being drought tolerant and Lok1, drought-sensitive [33,34,35]. The role of SA was studied in water stressed wheat plants through a range of developmental stages to assess different responses induced by SA in wheat plants. Yield parameters were studied to give an insight on the role of SA in productivity limiting drought stress. Proteomics study at vegetative and grain filling stage elucidated the network of proteins involved in SA regulation of water stress and ultimately their influence on the sink potential under yield limiting water stress.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted with two wheat (*Triticum aestivum* L.) varieties Kundan and Lok1 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). Seeds were manually sown in second week of November in pots at depth of 3–4 cm. Each set of experiment had 30 pots for biological replicates. Irrigation was maintained regularly till the seedling establishment i.e., 45 days after germination (DAG). Drought was maintained through controlling irrigation after 45 DAG till the harvesting stage. Two water stress levels were maintained according to Relative Water Content (RWC) of leaf (i) 50% RWC (ii) 75% RWC and recovery after 50% RWC through rehydration (RH). The control of well watered, severe stress (50%), moderate stress (75%) and rehydration of SA treatment were also maintained through experiment.

CW = Control well watered; CS1 = Control 75% stressed; CS2 = Control 50% stressed; CR = Control Rehydrated.

TW = SA treated well watered; TS1 = SA treated 75% stressed; TS2 = SA treated 50% stressed; TR = SA treated Rehydrated.

Moisture levels were kept constant by supplying water using a balance/watering set-up. Salicylic acid (SA) (0.5 mM) was applied through foliar spray to plants (control, water stressed and rehydrated) at regular intervals of 15 days starting from 45 DAG till harvesting. A set of plants of each treatment without SA application was also maintained.

2.2. RWC and soil moisture measurement

RWC of the leaves was determined as (FW-DW)/(TW-DW)× 100, where FW = Fresh weight; DW = Dry weight; TW = Turgid weight of the leaf after equilibration in distilled water for 24 h. Soil Moisture was measured through Soil moisture meter (ICT International Pvt. Ltd. Australia) in 5 pots of stressed and rehydrated plants after every 2 days. Measurements were performed in five replicate plants randomly selected from different pots of each set after every 2 days. The quantity of water to be provided for maintaining the RWC was decided through measuring both RWC and soil moisture before and after the supply of different amounts of water to plants. This preliminary experiment was performed five times for 10 days and every time with three different plants from different pots.

2.3. Plant growth and yield

Plants were harvested for biomass analysis at vegetative phase (8–9 weeks of growth) and flowering stage (after heading initiation) in five replicates. The final harvest (full maturity) of all remaining plants was performed for studying yield parameters. Leaf samples at both stages were also stored in Liq. N_2 and placed at −80 °C till further analysis. Root and shoot biomass were recorded after drying the plants in oven at 80 °C for 96 h. Spikelet number per inflorescence, inflorescence number per plant and inflorescence weight per plant and yield parameters like number of seeds per plant, grain weight per plant, and thousand grain weight and harvest index were also studied. Harvest index was calculated by following formula:

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

2.4. Leaf gas exchange and fluorescence analysis

The light-saturated rate of CO_2 assimilation (Asat), stomatal conductance (g_s), maximal efficiency of PSII (Fv/Fm) and intercellular [CO_2] (C_i) were estimated at a photosynthetic photon flux density (PPFD) of 1200 μmol m^{-2} s^{-1} using LiCOR model 6400 (Lincoln, Nebraska, USA) equipped with CO_2 control modules and LED light sources. Measurements were made on fully expanded leaves two leaves down from the youngest expanding leaf after 8–9 weeks of growth for vegetative phase and 12–13 weeks of growth for flowering phase. All measurements were made on warm and clear days between 0800 h to 1000 h. Leaf temperature was maintained at 25 °C and water pressure deficit between 1.0 to 1.5 kPa. Sample cell H_2O and flow rate were 20 mmol H_2O mol^{-1} and 500 μmol s^{-1}, respectively. Water use efficiency (WUE) was calculated by the ratio of A_N to transpiration rate (E). Three plants from different pots of each set were randomly selected for measurements.

2.5. Biochemical analysis

2.5.1. Antioxidative enzymes

Superoxide dismutase (SOD) activity was assayed using the photochemical NBT method [36] based on SOD's ability to inhibit the

reduction of nitro blue tetrazolium (NBT) to form formazan by superoxide. Catalase (CAT) activity was assayed by measuring the decrease in absorbance at 240 nm due to utilization of H_2O_2 [37]. The rate of hydrogen peroxide-dependent oxidation of ascorbic acid was estimated to study ascorbate peroxidase (APX) activity [38]. The Glutathione reductase (GR) activity was assayed by following the increase in absorbance at 412 nm when 5,50-dithiobis-(2-nitro-benzoic acid) (DTNB) was reduced by glutathione to form TNB [39].

2.5.2. Antioxidants and lipid peroxidation

Reduced ascorbate (ASA), dehydroascorbate (DHA) and total ascorbate were determined by following the method of [40]. Total glutathione, oxidized glutathione (GSSG) and reduced glutathione (GSH) were measured according to [41]. The level of lipid peroxidation in the leaf tissue was measured as content of malondialdehyde (MDA) equivalents using the 2-thiobarbituric acid (TBA) method [42].

2.6. Metabolomic compound determinations

2.6.1. Starch, total soluble sugars and proline content

Starch was extracted following the perchloric acid method described by Whelan [43] and was assayed using Iodine [44]. The method of Cross et al. [45] was followed for the extraction of total soluble sugars and proline from plant tissue. The proline content was assayed through Ninhydrin method. Anthrone method was used for total soluble sugars assay. Proline content was determined from standard curve for proline over a known concentration range and glucose standard used for total soluble sugar assay.

2.7. Proteomic characterization

2.7.1. Leaf 2DE and image analysis

Total soluble proteins were extracted from leaves of each treatment according to the method of Deeba et al. [46]. Proteins were extracted initially with extraction buffer 50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 500 mM thiourea and 0.5% 2-mercaptoethanol (BME) after grinding leaf sample in Liq N_2 followed by overnight TCA (trichloroacetic acid)-acetone precipitation at —20 °C and acetone washing. Second extraction of dried protein pellet was done after acetone wash by suspending pellet in buffer containing 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. Dried pellet was 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 with 7 cm IPG strips, pH 4–7 in Ettan IPGphor3 unit (GE Healthcare) in triplicates for each treatment. The IPG strips were loaded with 120 μg protein by overnight rehydration with total 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. After rehydration, focusing was performed under following conditions: 250 V Step for 1 h, 500 V Step for 1 h, 1500 V Step for 2 h, 4000 V Gradient for 2 h and 6000 V Step for 12000 Vh to reach total of 21 kVh. Consequently, equilibration of strips was performed in a 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 but with 2.5% (w/v) iodoacetamide replacing DTT. The second dimension was run in Mini-PROTEAN Tetra Cell by BioRad using 7 × 8 cm homogeneous SDS-PAGE gels of 16% T and 5% C. Electrophoresis was performed at constant Voltage 200 V in standard Tris-Glycine running buffer. Gels were stained with 0.5% Brilliant Blue G-250 for 45 min then destained in solution containing methanol, water and acetic acid (45:45:10 v/v) for 45 min followed by overnight destaining in Milli-Q.

Image analysis was performed using ImageMaster 2D Platinum 7.0 for differential protein analysis on the basis of relative volume (% volume) as increased or decreased (1.5 fold or more). Different statistical analysis was also performed as facilitated by the software.

The identification of protein spots was done through Mass spectrometry (MS) using 4800 *Plus* MALDI TOF/TOF Analyzer (ABSciex, USA). MS analysis was performed over sample processed and digested according to Koistinen et al. [47]. The selected spots were excised and destained 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 then trypsin digested overnight by using modified grade Trypsin (Promega, USA) in 1:20 ratio at 37 °C. Peptides were extracted thrice with 50% acetonitrile containing 1% Tri-fluoroacetic acid (TFA). The recovered peptides were concentrated to a final volume of 5 μl and analyzed. The tryptic peptides were dissolved in 5 mg/ml cyano-4-hydroxycinnamic acid consisting of 50% ACN and 0.1%TFA. The mono isotopic peptide masses obtained from MALDI-TOF were analyzed by the 4000 Series Explorer software version 3.5. The signal to noise ratio of 50 was the minimal criterion to define mass peaks. The MS spectra to acquire peptide mass fingerprint (PMF) were recorded in reflector mode in a mass range of 800–4000 with a focus mass of 2100. MS used ABI calibration mixture (CalMix) for calibration. MS spectra were obtained with 500 laser shots per spectrum. Up to twenty-five of the most intense ion signals were selected as precursors for MS/MS acquisition, including the trypsin autolysis peaks and the matrix ion signals. The peptides from the individual PMF spectra that exceeded a signal-to-noise ratio of 20 and passed through a mass exclusion filter were subjected to fragmentation analysis. MS/MS spectra were acquired with 1200 laser shots per fragmentation spectrum. The peak matching parameters were as follows: Min S/N: 20; mass tolerance: 0.5 *m/z*; min peaks to match reference masses, 4; and max outlier error, 11 ppm. Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed using the Mascot software (http://www.matrixscience.com) against NCBInr databases (Viridiplantae). The search parameters were as follows: trypsin cleavage (one missed cleavage allowed), carbamidomethylation as a fixed modification, methionine oxidation as a variable modification, peptide mass tolerance of 100 ppm, and MS/MS fragment mass tolerance of 0.2 Da. A peptide charge of 1+ was considered significant. Credible results for the MALDI-TOF/TOF MS were the proteins with a high protein score, confidence interval (C. I.%) of above 95% and similar molecular mass (Mr) and isoelectric point (pI) as experimental Mr and pI.

2.7.2. Western blot analysis

Leaf tissues were ground in liquid N_2. 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 benzamidine and 2 mM amino-*n*-caproic acid. Protein estimation was carried out by Bradford method. 7 μg of protein samples were resolved on 10% 1D-PAGE and blotted onto PVDF membrane at 16 V overnight. The blotting membrane blocked for 1 h in Tris buffered saline tween (TBST) buffer (pH 7.5) containing 1% bovine serum albumin (BSA). The membranes were probed by polyclonal primary antibodies from Agrisera, Sweden against AtpB (AS05 085), GS2 (AS08 296), PsbR (AS05–059), PRK (AS09 464) and FBPase (AS08 294) proteins at recommended dilution for 2 h in TBST containing 0.25% BSA. In the next step, HRP conjugated cross-reactive secondary antibody (1:20,000 dilutions) was incubated in 0.25% BSA containing TBST buffer. Blot was developed with HRP chromogenic substrate (TMB) for 5–10 min to visualize reactive bands. Densitometry analysis performed through Image Quant TL 7.0 software to study expression pattern.

2.8. Statistical analysis

All experiments were repeated three times independently. Spot intensities of differential proteins in a 2D gel were calculated from three

spots in three replicate gels. Growth, biochemical, physiological and yield parameters were statistically analyzed using a one-way analysis of variance (ANOVA) and post hoc Duncan's test to determine significant differences among group means at $p \leq 0.05$ level. The error for fold change was calculated for each differential spot. Linear Mixed Model ANOVA was performed to study the main effects and interactions of treatments (control, stress, rehydration with and without SA), variety and sampling time (vegetative, flowering and harvesting) on different parameters using SPSS 16.0. Principal component analysis (PCA) using XL-STAT was performed on morphological, physiological and biochemical parameters. k-Mean clustering, hierarchical clustering and heat map generation of proteins performed through Multi Experiment Viewer (MeV).

3. Results

3.1. Principal component analysis

The first principal component (PC1), which explained 35.48% of the variance, can be interpreted as biochemical response of plants to drought and its interaction with SA during vegetative stage. The 9 variables out of 21 correspond to the main loadings in PCA explaining most of the variance for the PCA-Axis 1. These variables were involved in biochemical response including enzymes APX, GR and SOD; antioxidant glutathione (GSH + GSSG; GSH), metabolites proline, total soluble sugars and starch (Root and Leaf). The scores in PC1 were dominated by tolerant (Kundan) variety under interaction (50 + SA and 75 + SA) and TS2 of Lok1. The second principal component (PC2), which explained 23.91% of the variance, was mainly loaded with physiological parameters and few biochemical parameters, e.g., chlorophyll content, A_N, F_v/Fm and g_s, reduced ascorbate and MDA equivalents. The water stress + SA showed significantly different responses as different clusters were observed through PCA. The control and rehydrated plants of both the varieties responded in similar pattern under SA treatment (Fig. 1A). Water stressed Kundan plants (CS2 and CS1) were clustered together with SA treated stressed Lok1 plants (TS2 and TS1). The biplot of flowering stage showed two principal components responsible for 52.43% variance (Fig. 1B). The PC1 was dominated by tolerant variety with the factor loadings of antioxidant glutathione level, antioxidative enzymes (APX and catalase) and total soluble sugars and explained 26.7% of the variance. The control, stressed and their interaction with SA behaved in similar way in both varieties. The scores plot also suggested a visible differentiation between the two varieties response grouping them in two visible clusters (Fig. 1B). PC2, explaining 23.75% of total variance, was largely determined by A_N, F_v/F_m, WUE, chlorophyll under rehydration having strong positive loadings demonstrating dominance of physiological variables in both varieties.

3.2. Yield

Among the yield parameters, number of seeds per plant and seed weight per plant were increased significantly (TRT-ANOVA $p < 0.001$) in SA treated plants of both varieties (Fig. 2A, B). Seed weight per plant was higher in tolerant variety than in sensitive variety under control and treatment (VR-ANOVA and TRT*VR-ANOVA $p < 0.001$). Seed starch content increased (TRT-ANOVA $p < 0.001$) significantly in TW, TS1, TS2 in both varieties (Supplementary Fig. S2B). Seeds of Kundan contained more starch than Lok1 seeds both under control as well as in treatment. Harvest index (HI) was also significantly increased (TRT-ANOVA $p < 0.003$) in SA treated Kundan and Lok1 plants (Fig. 2C).

3.3. Differential proteomics and functional characterization

Leaf proteomics revealed increased or decreased abundance of proteins under interactive effects of SA with water stress, rehydration and well watered condition in both varieties during two growth stages (Figs. 3–4 and Tables 1–2). Abundance of proteins was more at vegetative stage in both varieties, predominantly in Lok1, than at flowering stage (more in Kundan) (Tables 1–2). The number of identified proteins was 91 and 83 in Kundan at vegetative and flowering stage, respectively. In Lok1, 90 proteins were identified at vegetative stage and 46 proteins were identified at flowering stage. Identified proteins were functionally characterized and mostly belonged to the *Triticum* species (Supplementary Fig. S18 and Supplementary Table S5 Data set S5A and S5B). Proteins were characterized in 11 functional categories for Kundan and 10 functional categories for Lok1 (Supplementary Fig. S18). Photosynthesis and carbon metabolism were the two major dominating functional categories. The number of photosynthesis related proteins was 48 in Kundan and 37 in Lok1 (Tables 3 & 4). Thirty-eight proteins of Kundan and 31 of Lok1 were involved in carbon metabolism. Many proteins related to protein synthesis, assembly and degradation (21 in Kundan and 17 in Lok1) were also identified (Tables 3 & 4). The amino acid metabolism category had 16 proteins in Kundan and 17 in Lok1. Defense category had 17 and 12 proteins in Kundan and Lok1, respectively (Tables 3 & 4). There were 11 energy metabolism proteins in Kundan and 6 in Lok1. Nine proteins of Kundan and 6 proteins from Lok1 were categorized under redox signaling. Signal transduction category had 5 proteins from Kundan and 1 protein from Lok1.

3.4. k-Mean clustering

Clustering analysis of the relative abundance of identified proteins to water stress under SA treatment revealed 8 clusters in Lok1 and Kundan during both vegetative and flowering stages (Fig. 5). Clusters 1–4 (Supplementary Table S6 Dataset S6A-6D) and Clusters 5–8 (Supplementary Table S6 Dataset S6E-6H) represent groups of proteins with differential relative abundance at vegetative and flowering stage, respectively. k-mean clustering grouped proteins with similar pattern of abundance under treatment in same cluster. Cluster 1 had 34 proteins which showed higher abundance in TS2 followed by TS1, TW and TR. Cluster 1 represented SA responsive protein group mainly belonging to carbon metabolism (TK), photosynthesis (RuBisCO), and signal transduction (TCTP) in both varieties. FBPase, PPDK1, PGD (carbon metabolism); RA, Chla/bBP, FNR (photosynthesis); Hsps, LAP (protein synthesis, assembly and degradation) were proteins of Cluster 1 specific to Kundan. In Lok1, protein metabolism (EFs, PPI, GRP), amino acid metabolism (GS, SAM) and defense (LEA, CysPrx) were three additional categories. Cluster 2 had 61 proteins, mainly positively regulated on rehydration under SA treatment (TR), then in TS1 and TS2. Under well watered condition, SA treatment caused decrease in abundance of Cluster 2 proteins. Proteins responsive to drought stress and rehydration under SA treatment in Cluster 2 mainly involved in photosynthesis (RuBisCO, PSP), carbon metabolism (proteins related to photosynthesis, CA) and energy metabolism (ATPase) for both varieties. Photosynthesis protein TLP, carbon metabolism FBPase and redox signaling proteins TrxM and H were specific to Kundan in Cluster 2. Proteins specific to Lok1in Cluster 2 were Chla/bBP (photosynthesis); GS, CS, GABA (amino acid metabolism); TK, TPI, SBPase, UGPase (carbon metabolism) and 50S RP, Chaperons (protein synthesis, assembly and degradation). Most of the proteins out of 42 in Cluster 3 had positive regulation in TS1 then in TR. Cluster 3 also had some proteins mainly showing increased abundance in TW and decrease in TS2 plants. Cluster 3 represented SA responsive proteins under moderate stress and recovery categorized under photosynthesis (RuBisCO) and carbon metabolism (FBPase) category functioning to maintain high rate of photosynthesis and cytoskeleton (actin) in Kundan. Protein synthesis, assembly and degradation (Hsps, chaperons, PDX1, CSP), amino acid metabolism (CS, SAMS) and energy metabolism (ATPase) were major categories for Lok1 in Cluster 3. Differential regulation (mostly abundance) was observed in proteins of Cluster 4 in TW and TR conditions. Some other proteins in Cluster 4

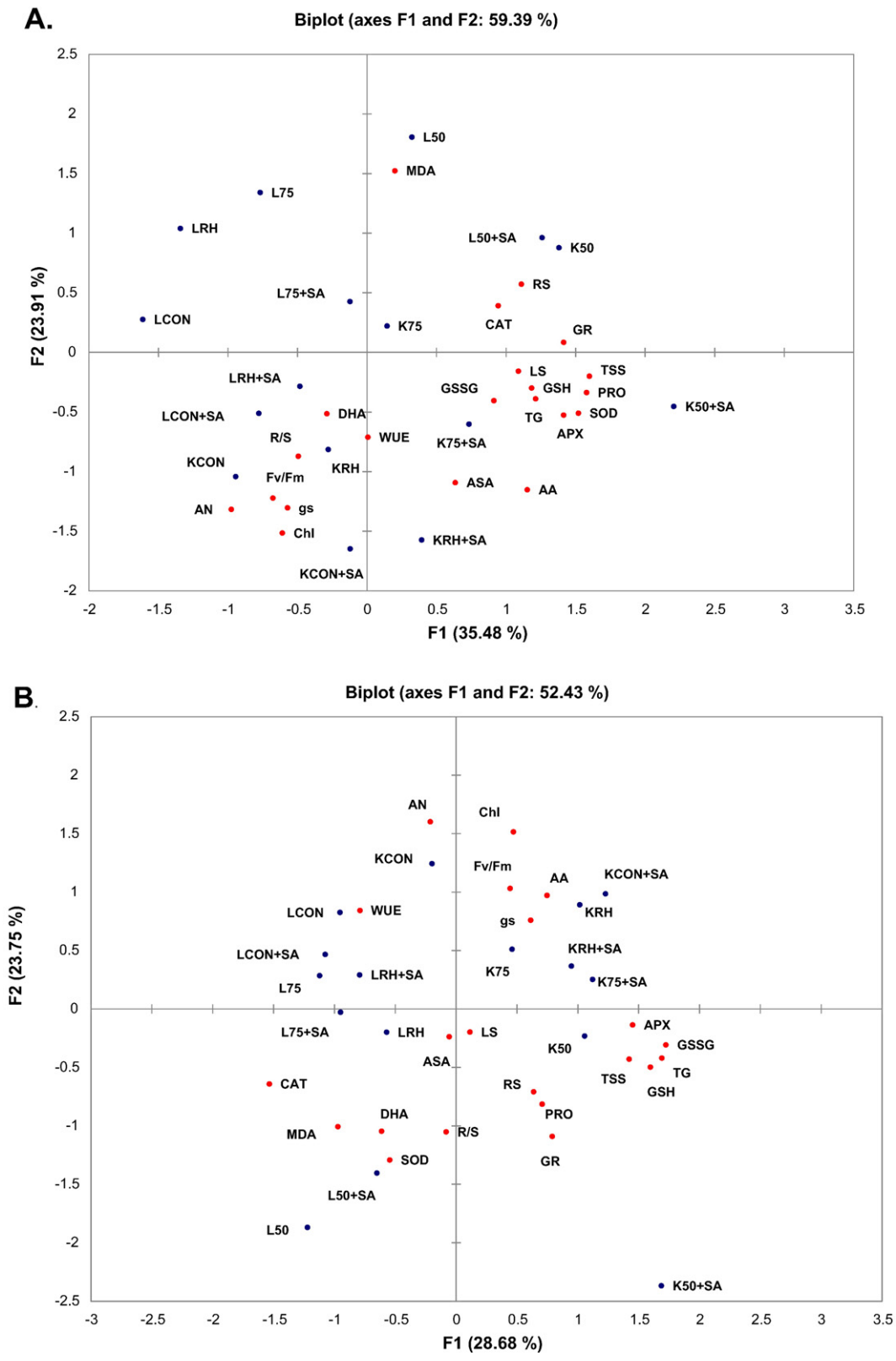

Fig. 1. PCA biplots showing two principal components causing maximum variance on the basis of morphological, physiological and biochemical variables under the interactive effect of drought and SA on wheat varieties. A. Vegetative stage B. Flowering stage. R/S = root to shoot ratio; APX = ascorbate peroxidase; GR = glutathione reductase; CAT = catalase; SOD = superoxide dismutase; AA = total ascorbate; ASA = reduced ascorbate; DHA = dehydroascorbate; TG = total glutathione; GSSG = oxidized glutathione; GSH = reduced glutathione; LS = leaf starch; RS = root starch; TSS = total soluble sugars in leaf; PRO = proline in leaf; MDA = malondialdehyde; Chl = chlorophyll; AN = rate of photosynthesis; gs = stomatal conductance; Fv/Fm = PSII maximal efficiency; WUE = water use efficiency. L represents Lok1 variety and K represents Kundan variety. Well watered control = LCON and KCON; control severe stressed = L50 and K50; control moderate stress = L75 and L75; control rehydration = LRH and KRH; well watered treated = LCON + SA and KCON + SA; severe stress treated = L50 + SA and K50 + SA; moderate stress treated = L75 + SA and K75 + SA; rehydration treated = LRH + SA and KRH + SA.

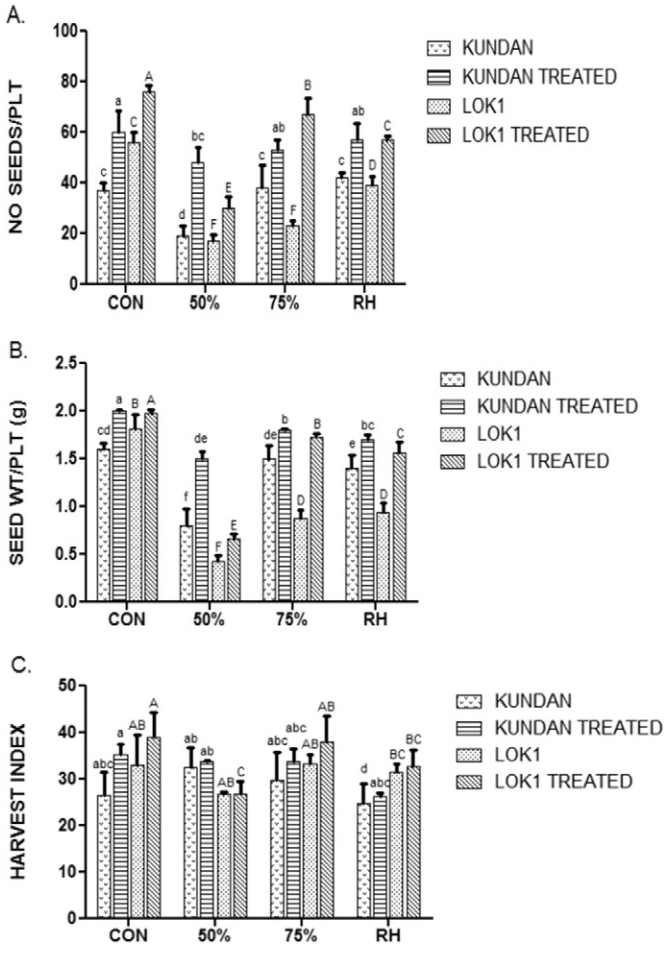

Fig. 2. Yield parameters A. Number of seeds per plant; B. Seed weight per plant; C. Harvest index (HI) under interaction of drought with SA in Kundan and Lok1. Bar represents mean ± standard deviation ($n = 3$), letters (ABC) and (abc) represents level of significance for Lok1 and Kundan respectively through one way ANOVA post hoc Duncan's test ($p \leq 0.05$).

showed increase in TS2 and some decrease in TS2 and TS1. Proteins mainly responsible for recovery from drought stress under SA of Cluster 4 functionally categorized under photosynthesis (RuBisCO, RA, TLP, OEE1, Cytb6-f), protein synthesis, assembly and degradation (RBP, GRP, 50S RP, PPI), amino acid metabolism (CS) and redox balance (redox signaling and defense; TrxM, SOD, Prxs, SSB) in Kundan. Cluster 5 had 16 proteins mostly with increased abundance in TR and decrease in TS2 plants. RuBisCO protein of photosynthesis category was common among both varieties in Cluster 5. Proteins related to recovery from severe water stress under SA during later growth stage of Cluster 5 categorized in photosynthesis (PSI subunit VII) and amino acid metabolism (GS) specifically for Lok1. Proteins of Cluster 5 specific to Kundan were categorized under redox signaling (TrxM) and defense (GST). Cluster 6 consisted of 49 proteins with decreased abundance in TS1 and TR plants as compared to increase in TW and TS2. Proteins involved in photosynthesis and related carbon metabolism (RA, TLP, OEE2, PRK, FBPase), amino acid metabolism (GS, SAM) and protein metabolism (EFs, chaperons) were grouped in Cluster 6 for Kundan. Photosynthesis (RuBisCO), carbon metabolism (SBPase, PGD, TK) and energy metabolism (ATPase) were major functional categories for Lok1 in Cluster 6. Proteins showing negative influence of SA on their abundance in TS1 were grouped in Cluster 7 and categorized in all functional categories for Kundan and only amino acid (CS, GABA) and carbon metabolism (FBPase, PGPase) for Lok1. Some proteins out of 36 in Cluster 7 were increased in TW, TS2 and TR. Cluster 8 had group of proteins negatively regulated in TW and TS2 but some showed higher abundance in TS1 and TR. Protein categorized in all functional categories (photosynthesis, RuBisCO, OEE, Cytb6-f; carbon metabolism, CA; energy metabolism, ATPase; amino acid metabolism, GS; defense, SOD, GLP; redox signaling, NDPK2) excluding protein metabolism category in both varieties were grouped under Cluster 8. Clusters 6, 7 and 8 grouped SA responsive proteins which were responsible for stress tolerance and increased sink strength (Fig. 6).

3.5. Western blot

Western blot of selected candidate proteins were performed for validating the results of 2DE gel analysis (Supp. Fig. S19). We have selected ATPase beta (AtpB), GS2, Photosystem II protein (PsbR), PRK and FBPase proteins based on their roles on the cellular physiology of SA and drought interaction. Western blot results verified their expression in treated conditions as reported through MS analysis (Supplementary Fig. S19). The expression pattern was found to be similar under all conditions as observed through 2D gel electrophoresis (Supplementary Fig. S20).

4. Discussion

In present study, drought stressed wheat plants showed diminished growth and yield while plants treated with 0.5 mM SA showed enhanced biomass accumulation, improved physiological performance, less lipid peroxidation, high antioxidative enzyme activities and better yield. Differential proteomics substantiated the role of SA in maintaining the plant vigor under water limiting environment. The interplay of SA and redox signaling have role in conferring drought tolerance. Kundan variety responded better to SA treatment than Lok1 variety. These results indicate that SA treatment positively modified regulatory pathways resulting in better growth and enhanced drought tolerance in wheat varieties without compromising yield. This is the first field study demonstrating the role of exogenous SA in providing drought tolerance, influencing the protein expression regulating physiology, metabolism and defense throughout the growth stages of wheat. The regulatory pathways studied during anthesis depicted the positive influence of SA in ameliorating yield limitations by improving sink potential.

4.1. Plant growth

Plant growth and biomass allocation are two important aspects negatively impacted under drought stress [48]. Alleviation of drought stress by SA through improved carbon assimilation and increasing plant biomass has been reported [27]. Biomass allocation was found to be more towards roots of Lok1 and more towards shoot in Kundan (Supplementary Fig. S1). Similar pattern of biomass allocation in drought stressed sensitive and tolerant wheat plants [23] and groundnut [49] under SA treatment has been reported. Kundan plants having well developed root system allocated more biomass towards shoot through increased C assimilation under SA treatment. SA induced changes in starch content of root and leaves in both varieties supported the biomass allocation pattern (Supplementary Fig. S2). Tolerant variety showed accumulation of starch in leaves of TS1, TS2 and TR plants due to decreased abundance of TK (spots K27, K82; Cluster 1) relieving the inhibition from starch biosynthesis positively affecting the water use efficiency [50,51,52]. The abundance of TK (spots L1, L15, L79), UGPase (spot no L73) and TPI (spots L42, L43) (Cluster 2) shifted the C metabolism more towards accumulation of soluble sugars in Lok1 (TS1, TS2 and TR), supported by earlier findings [53,54,55,56]. Plant growth and osmotic adjustment were maintained by SA through increased soluble sugars content as reported earlier [57,58]. The higher starch in SA treated leaves of both varieties as compared to their control was explained

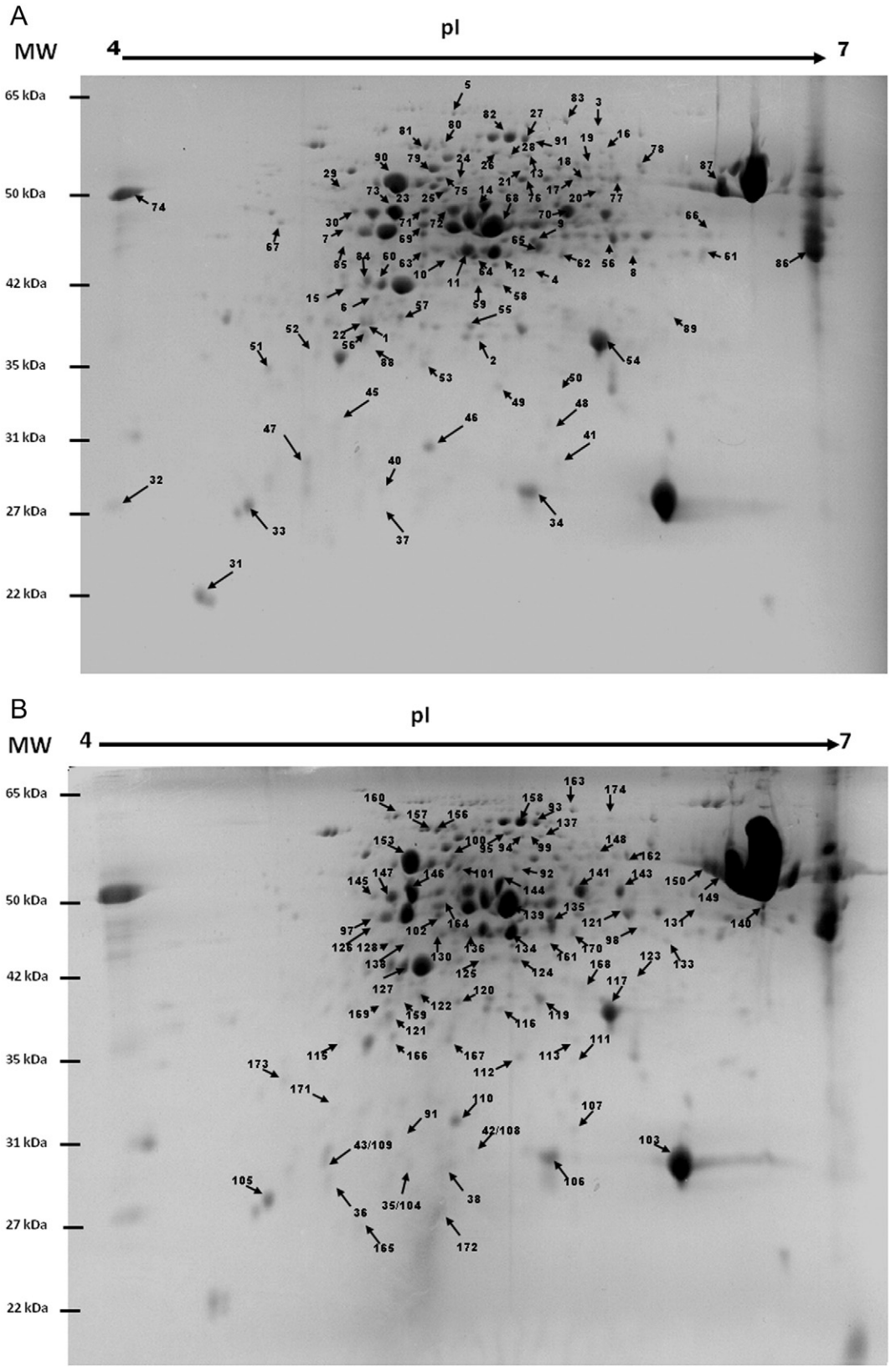

Fig. 3. Representative two dimensional gel of Kundan variety under A. Control severe stressed (CS2); B. Treated severe stressed (TS2). Isoelectric focusing was performed over 4–7 pI range immobiline strip of 7 cm and second dimension was performed through 16% SDS-PAGE in Tris-glycine buffer and stained with coomassie brilliant blue G.

through the abundance of PGD (spots K20, L4; Clusters 1–2) providing glucose-1-phosphate for starch synthesis [59,60]. The carbohydrate metabolism enzymes abundance involved in glycolysis like GADPH (spots K70, L67), PGM (spots K28, K94) and Krebs cycle e.g., mMDH (spots K61, L55) throughout the growth stages corroborated biomass accumulation (Clusters 1–4, 6–8).

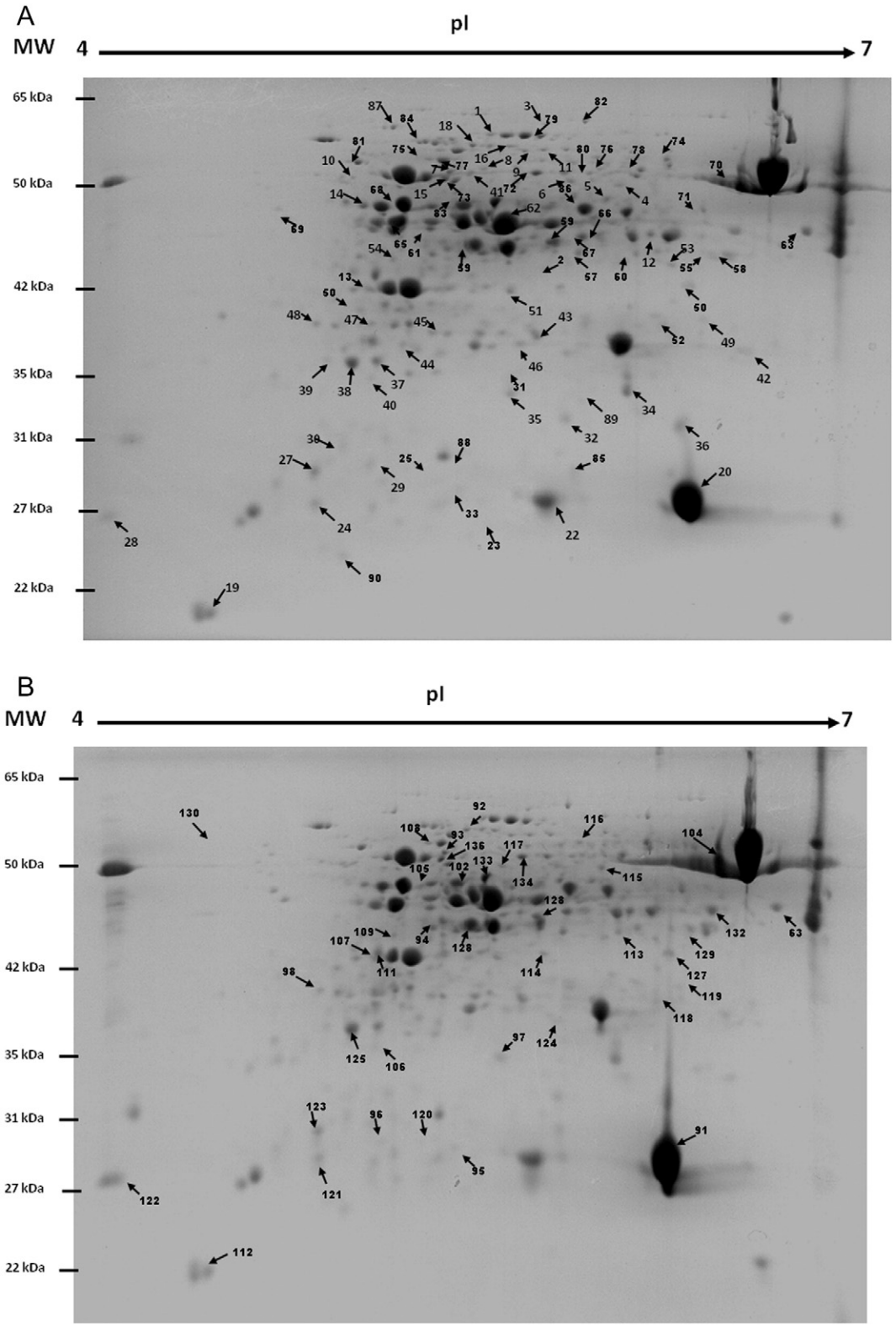

Fig. 4. Representative two dimensional gel of Lok1 variety under A. Control moderate stressed (CS1); B. Treated moderate stressed (TS1). Isoelectric focusing was performed over 4–7 pI range immobiline strip of 7 cm and second dimension was performed through 16% SDS-PAGE in Tris-glycine buffer and stained with coomassie brilliant blue G.

4.2. Physiological responses

A major effect of drought is reduction in photosynthesis and impaired photosynthetic machinery including stomatal control of the CO_2 supply, electron transport and carbon reduction cycle [61]. In the present study, SA alleviated photosynthetic limitation under water stress (Supplementary Fig. S9). The PC2 responsible for causing variability under drought and its interaction with SA mainly showed physiological variable loadings at both stages (Fig. 1). Increased A_N was observed in SA treated plants (TW, TS1, TS2 and TR) with pronounced increase in

Table 1
Number of leaf proteins in Kundan showing differential abundance under interactive effect of SA with well watered, water stressed and rehydrated conditions at both stages of development.

		Vegetative stage[a]								Flowering stage[a]							
Kundan		Control[b]		75%[b]		50%[b]		RH[b]		Control[b]		75%[b]		50%[b]		RH[b]	
Differential abundance[c]		65		54		64		86		64		77		64		59	
Increased[d]		59		29		47		77		50		18		40		30	
Decreased[e]		6		25		17		9		30		59		24		29	
Identified[f]	Increased ↑	53	52	42	25	52	38	54	49	41	24	54	10	39	24	40	25
	Decreased ↓		1		17		14		5		17		44		15		15

[a] Two growth stages of sampling: vegetative and flowering.
[b] Different conditions: control-well watered, 75%–75% RWC (moderate stress, S1), 50%–50%RWC (severe stress, S2), 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.

Kundan than Lok1. The higher abundance of RuBisCO (spots K34, K38, K44, K74, K87, L20, L22, L49, L7; Clusters 1–4), RuBisCO activators like PRK (spots K7 and K97) and RA (spots K69, K71, K72, L62, L83) and RuBP regenerating enzymes SBPase (spot L65) and FBPase (spots K9, K65, K85, L10, L60) positively influenced photosynthesis in both varieties. SA enhanced g_s increasing internal CO_2 concentration corroborated with increased abundance of CA (spots K89, L38, L39; Clusters 2 and 3) improving the carboxylation efficiency of RuBisCO under drought. SA protected the photosynthetic apparatus from drought generated oxidative stress [62,63] through abundance of OEE1 and OEE 2 (spots K54, K60, L40), PSP (spots K23, L61, L94), FNR (spots K12, K98), Cytb6-f (spots K48, L29, L89, L96) Chla/bBP (spots K55, L44, L45) and TLP (spots K45, L28). Stress acclimation and maintenance of physiology require high energy which was fulfilled by abundance of ATP synthase subunits (spots K78, K90, L34, L74, L93; Clusters 1–4) under SA [64,65]. Budak et al. [66] findings supported the increased energy metabolism in sensitive Lok1 to cope with stress. While in tolerant Kundan most of these proteins were decreased explaining less oxidative stress under SA. Western blot analysis confirmed the role of proteins related to photosynthesis and energy i.e., FBPase, ATPase and PSP in SA mediated drought responses (Supplementary Figs. S19–S20). Improved water use efficiency (WUE) observed in both varieties under all conditions are deemed as effective strategy for SA induced drought tolerance (Supplementary Fig. S12). SA improved physiological attributes are reported in different plants under drought [67,68,69]. After anthesis leaves play role of organ for storing and transporting assimilates to developing grains [70]. In accordance, we found decrease in physiological attributes at flowering as compared to vegetative stage. SA maintained the higher physiological performance as compared to respective control in TC, TS1, TS2 and TR Kundan plants and TC, TS1 and TR Lok1 plants at anthesis positively affecting sink potential (Supplementary Fig. S9–S12). The decline in abundance of RuBisCO large subunit (spots K150, L104, L135), PSP (spot K102) and other photosynthesis related proteins (spots L96, K98, K117, K127) was closely associated with the N mobilization from leaves to meet high demand of developing seeds (Clusters 6–8) [71,72]. Simultaneously, abundance of RA (spot K139), SBPase (L99) and RBP (spots L92, K156; Clusters 5–6) protected RuBisCO from denaturation/degradation and maintained the activity of residual RuBisCO explaining increased physiological performance under SA during N mobilization at flowering stage [71]. The interactive effects of SA and water deficit in TS2 plants of Lok1 induced early senescence as drought avoidance mechanism decreasing all physiological parameters and chlorophyll at flowering stage [73,74]. The physiological traits were responsible for SA induced tolerance in rehydrated plants at the level with well watered plants and observed to be high in tolerant variety (Fig. 1). Physiological functions were maintained in TR Kundan plants during both the growth stages while in Lok1 during vegetative stage only. At flowering stage, physiological recovery could not occur in TR Lok1 plants due to senescence. Abundance of RuBisCO subunits (spots K74, K76, K67, K38, K108, K113, L95), CA (spots K89, K123, L38), FBPase (spots K85, K126, L60), SBPase (spots K138, L65, L99), RA (spots K68, K71, L40) and PRK (spots K7, K97) maintained the photosynthetic rate on rehydration. OEE (K54), PSP (spots K23, L61) and ATPase (spots K75, K78, L34, L74) negated the adverse effect of water stress on photosynthetic apparatus and provided energy for recovery under SA treatment. Many other differential proteomics studies support our results [75,76,77].

4.3. Defense responses

SA induces different defense responses in plants against oxidative stress generated under drought [15,16,19]. These responses comprised of effective antioxidant system, osmoregulation and abundance of defensive proteins. PCA analysis revealed role of efficient antioxidant

Table 2
Number of leaf proteins in Lok1 showing differential abundance under interactive effect of SA with well watered, water stressed and rehydrated conditions at both stages of development.

		Vegetative stage[a]								Flowering stage[a]							
Lok1		Control[b]		75%[b]		50%[b]		RH[b]		Control[b]		75%[b]		50%[b]		RH[b]	
Differential abundance[c]		51		75		73		61		61		19		12		22	
Increased[d]		19		68		66		48		38		9		5		12	
Decreased[e]		32		7		7		13		23		10		7		10	
Identified[f]	Increased ↑	33	19	74	68	40	36	41	38	40	23	14	7	6	2	13	8
	Decreased ↓		16		6		4		3		17		7		4		5

[a] Two growth stages of sampling: vegetative and flowering.
[b] Different conditions: control-well watered, 75%–75% RWC (moderate stress, S1), 50%–50%RWC (severe stress, S2), 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.

Table 3
List of identified differentially regulated leaf proteins of Kundan 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]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change ± S.E.[i] (SA treated/control) CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Photosynthesis[k]									
Ferredoxin-NADP(H) oxidoreductase	K8/K98[a]	*Triticum aestivum* gi|20302473	416	6.9/40.5	8(5)/27	1.5 ± 0.04/2.5 ± 0.01	1 ± 0.1/−2.4 ± 0.1	−2 ± 0.2/−2 ± 0.3	−1.6 ± 0.04/1 ± 0.1
	K12[b]	*Triticum aestivum* gi|20302471	342	8.3/39.2	9(2)/30	1.9 ± 0.03/–	1 ± 0.05/–	1.9 ± 0.2/–	1 ± 0.1/–
RuBisCO large subunit	K18/K150[a]	*Triticum aestivum* gi|14017580	1350	6.2/53.4	17(12)/42	1.6 ± 0.1/−1.6 ± 0.2	1 ± 0.05/−2 ± 0.02	−1.9 ± 0.2/1 ± 0.5	1 ± 0.1/−10 ± 0.1
	K74[b]	*Triticum aestivum* gi|14017580	208	6.2/53.4	8(1)/20	1 ± 0.06/–	1 ± 0.02/–	2.7 ± 0.2/–	2 ± 0.2/–
	K76/K152[a]	*Needhamiella pumilio* gi|1750360	141	5.8/53	3(1)/8	1.7 ± 0.05/1.1 ± 0.2	−1.5 ± 0.1/−3 ± 0.3	1.1 ± 0.1/1 ± 0.001	3.2 ± 0.04/5.8 ± 0.3
	K87/K149[a]	*Psathyrostachys fragilis* gi|31087919	525	6.2/53.7	8(4)/25	1 ± 0.05/1 ± 0.1	1.1 ± 0.1/−2.5 ± 0.06	2 ± 0.05/2.5 ± 0.01	9 ± 0.6/−2.6 ± 0.4
	K173[c]	*Psathyrostachys fragilis* gi|31087919	424	6.2/53.7	6(3)/23	–/−4 ± 0.1	–/1 ± 0.05	–/−1.6	–/1 ± 0.02
Ribulose-bisphosphate carboxylase	K19/K155[a]	*Melampyrum pratense* gi|1490370	193	6.1/53.5	5(1)/11	1.5 ± 0.06/1.5 ± 0.1	1.1 ± 0.1/−7.1 ± 0.2	1 ± 0.1/1.3 ± 0.2	1 ± 0.004/1.3 ± 0.2
NADP-thioredoxin reductase C precursor	K21[b]	*Hordeum vulgare* gi|164598928	366	5.4/52.4	8(3)/19	1 ± 0.1/–	1 ± 0.1/–	−1.8 ± 0.2/–	1 ± 0.004/–
Chlorophyll a/b binding protein	K22/K169[a]	*Triticum aestivum* gi|302566696	145	5.1/28.4	3(2)/9	2.8 ± 0.3/1.6 ± 0.01	−1.5 ± 0.1/1 ± 0.1	1 ± 0.4/1 ± 0.04	1 ± 0.04/1 ± 0.1
	K55[b]	*Triticum urartu* gi|474121685	326	8.7/29.3	6(3)/39	1.8 ± 0.2/–	1.7 ± 0.001/–	2 ± 0.2/–	1.6 ± 0.2/–
Photosystem II stability/assembly factor HCF136	K23/K102[a]	*Brachypodium distachyon* gi|721612762	537	8.9/43.1	6(5)/20	1 ± 0.2/1 ± 0.1	1 ± 0.03/−2.6 ± 0.3	1.6 ± 0.2/1 ± 0.1	2.1 ± 0.03/1 ± 0.2
Thylakoid lumenal 17.4 kDa protein	K32[b]	*Brachypodium distachyon* gi|721670743	66	8.6/25.9	2(0)/11	1.9 ± 0.2/–	3.3 ± 0.01/–	2.9 ± 0.2/–	3 ± 0.7/–
	K45[b]	*Oryza sativa* Japonica Group gi|1002298925	90	8.6/25.9	2(0)/11	1.7 ± 0.2/–	1.5 ± 0.1/–	1.5 ± 0.2/–	1.9 ± 0.1/–
RuBisCO small subunit	K34/K106[a]	*Triticum aestivum* gi|11990901	643	8.8/19.7	11(8)/62	1.6 ± 0.3/1 ± 0.1	1 ± 0.2/−2.4 ± 0.1	1 ± 0.3/1 ± 0.04	1.3 ± 0.1/1 ± 0.1
	K38[b]	*Triticum aestivum* gi|82619	52	8.9/15.6	2(0)/11	2.7 ± 0.3/–	1 ± 0.03/–	1.5 ± 0.1/–	2.6 ± 0.01/–
	K42	*Triticum aestivum* gi|132107	162	5.8/13.3	4(1)/30	1.5 ± 0.2/–	1.2 ± 0.02/–	3.9 ± 0.1/–	1.3 ± 0.2/–
	K44/K108[a]	*Triticum aestivum* gi|11990893	174	9.1/20	5(0)/20	2.8 ± 0.3/1.1 ± 0.01	1.8 ± 0.2/1.1 ± 0.1	1 ± 0.2/2 ± 0.001	1 ± 0.01/6 ± 0.8
	K99[c]	*Triticum aestivum* gi|132107	143	5.8/13.3	4(1)/30	–/−3.5 ± 0.4	–/1.6 ± 0.1	–/−2.1 ± 0.05	–/1.5 ± 0.2
	K103[c]	*Hordeum vulgare* gi|132087	174	8.9/19.7	4(1)/20	–/34.1 ± 0.01	–/−49.8 ± 3.9	–/21.6 ± 0.6	–/1 ± 0.05
	K113[c]	*Triticum aestivum* gi|11990897	325	8.8/19.7	7(3)/35	–/1 ± 0.004	–/1 ± 0.06	–/1 ± 0.01	–/3.9 ± 0.3
Cytochrome b6-f complex iron-sulfur subunit	K48/K111[a]	*Triticum aestivum* gi|68566191	265	8.5/24.1	7(2)/33	1.7 ± 0.2/−2.6 ± 0.2	1 ± 0.05/6 ± 0.7	−4.6 ± 0.4/−4.4 ± 0.4	3 ± 0.02/2.6 ± 0.1
Thylakoid lumenal 19 kDa protein	K53/K167[a]	*Brachypodium distachyon* gi|357148370	99	5.6/25.9	2(1)/11	1 ± 0.1/1 ± 0.003	1 ± 0.4/1 ± 0.006	1 ± 0.03/1.7 ± 0.1	1.8 ± 0.02/1 ± 0.1

(continued on next page)

Table 3 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change ± S.E.[i] (SA treated/control)			
						CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Oxygen-evolving enhancer protein	K54/K117[a]	*Triticum urartu* gi|474077556	399	8.9/26	7(3)/35	1 ± 0.1/1.7 ± 0.005	1 ± 0.4/−1.8 ± 0.1	1 ± 0.01/1.5 ± 0.02	1.6 ± 0.2/−4.9 ± 0.1
	K60/K127[a]	*Triticum urartu* gi|474352688	127	5.7/34.6	2(1)/14	2 ± 0.1/1 ± 0.03	4 ± 0.03/−1.5 ± 0.1	3.6 ± 0.05/1 ± 0.002	1.7 ± 0.05/−2.6 ± 0.02
RUBISCO activase	K68/K139[a]	*Triticum urartu* gi|474153435	172	6.9/51.2	3(1)/10	1 ± 0.1/2 ± 0.03	1 ± 0.5/2 ± 0.2	1 ± 0.2/1.5 ± 0.0003	1.5 ± 0.03/1 ± 0.1
	K69/K164[a]	*Triticum aestivum* gi|671744544	226	5.6/45	7(2)/25	1.9 ± 0.1/1.3 ± 0.002	1 ± 0.2/1 ± 0.1	1 ± 0.2/−1.7 ± 0.2	−1.7 ± 0.2/1.3 ± 0.1
	K71[b]	*Triticum aestivum* gi|671744544	579	5.6/45	10(5)/25	1 ± 0.2/–	2 ± 0.02/–	2 ± 0.2/–	1.5 ± 0.1/–
	K72[b]	*Triticum aestivum* gi|671744544	371	5.6/44.8	9(3)/24	1 ± 0.2/–	1.5 ± 0.008/–	1 ± 0.1/–	1 ± 0.1/–
Carbon metabolism[k]									
Transketolase, chloroplastic	K1/K159[a]	*Triticum urartu* gi|474352176	263	5.4/69	10(4)/24	2.3 ± 0.1/1 ± 0.1	1 ± 0.002/−2.6 ± 0.1	1 ± 0.05/1.6 ± 0.01	1 ± 0.002/1 ± 0.1
	K27/K93[a]	*Tarenaya hassleriana* gi|729346302	186	6.6/81.4	5(2)/4	1.2 ± 0.2/−2.8 ± 0.5	1.8 ± 0.02/−3.2 ± 0.2	2 ± 0.2/1.5 ± 0.05	−1.6 ± 0.1/1 ± 0.2
	K82/K158[a]	*Aegilops tauschii* gi|326533372	425	5.4/69.4	12(4)/26	1.5 ± 0.2/−1.5 ± 0.001	−2.2 ± 0.5/−2.7 ± 0.07	2 ± 0.2/1.6 ± 0.001	1 ± 0.1/−1.6 ± 0.01
Phosphoglycolate phosphatase	K2/K116[a]	*Solanum lycopersicum* gi|460409293	193	8.3/40	3(3)/7	−2 ± 0.1/1 ± 0.02	−2.6 ± 0.07/1 ± 0.1	−2 ± 0.2/1 ± 0.005	1 ± 0.01/3 ± 0.2
	K84[b]	*Aegilops tauschii* gi|475589243	428	4.9/33.7	9(4)/36	1 ± 0.1/–	−3 ± 0.2/–	1.5 ± 0.1/–	1 ± 0.1/–
Pyruvate phosphate dikinase	K5[b]	*Aegilops tauschii* gi|475594808	189	6.5/141.7	8(0)/7	1 ± 0.1/–	2 ± 0.01/–	4.8 ± 0.2/–	2 ± 0.02/–
Phosphoribulokinase (PRK)	K7/K97[a]	*Triticum aestivum* gi|21839	150	5.8/45.4	6(0)/14	1.7 ± 0.05/4.5 ± 0.03	1.1 ± 0.01/1.1 ± 0.03	1.5 ± 0.03/2.5 ± 0.01	2.1 ± 0.005/2.1 ± 0.2
Fructose-bisphosphate aldolase (FBPase)	K9/K135[a]	*Triticum aestivum* gi|223018643	247	5.9/42.2	7(1)/33	1.2 ± 0.2/3.8 ± 0.1	2.7 ± 0.02/−2.2 ± 0.2	1.1 ± 0.2/1 ± 0.01	2 ± 0.3/1.3 ± 0.2
	K29[b]	*Triticum aestivum* gi|119745	253	5.2/45	6(3)/15	1 ± 0.1/–	1 ± 0.04/–	2.9 ± 0.2/–	1 ± 0.1/–
	K64[b]	*Triticum aestivum* gi|223018643	616	5.9/42.2	11(5)/35	1 ± 0.2/–	1 ± 0.08/–	1 ± 0.2/–	1.6 ± 0.2/–
	K65[b]	*Triticum aestivum* gi|223018643	1215	5.9/42.2	14(11)/42	1.5 ± 0.2/–	1 ± 0.04/–	3 ± 0.1/–	1 ± 0.2/–
	K66/K131[a]	*Triticum urartu* gi|473936969	623	8.5/70	9(6)/18	1.8 ± 0.2/1 ± 0.1	1 ± 0.01/−3 ± 0.7	1 ± 0.1/1 ± 0.03	1 ± 0.01/1.8 ± 0.3
	K85/126[a]	*Jatropha curcas* gi|802708115	84	8.4/42.9	2(0)/9	1 ± 0.1/−1.9 ± 0.5	2.5 ± 0.03/−2 ± 0.04	1 ± 0.1/1.2 ± 0.03	1.5 ± 0.04/1.9 ± 0.1
	K134[c]	*Triticum aestivum* gi|223018643	473	5.9/42.2	9(5)/28	–/1.5 ± 0.01	–/−2 ± 0.07	–/1 ± 0.003	–/−1.5 ± 0.1
6-phosphogluconate dehydrogenase, decarboxylating 1	K20/K148[a]	*Brachypodium distachyon* gi|357110692	240	5.6/52.9	6(2)/15	1.5 ± 0.08/1.5 ± 0.01	1 ± 0.2/1.5 ± 0.3	1 ± 0.2/2.6 ± 0.003	−1.5 ± 0.1/1.6 ± 0.1
UTP–glucose-1-phosphate uridylyltransferase (UGPase)	K25/K101[a]	*Triticum urartu* gi|473993048	759	5.8/51	11(8)/26	1 ± 0.3/1 ± 0.03	−2 ± 0.5/3.5 ± 0.2	−1.8 ± 0.1/1 ± 0.07	−2 ± 0.04/−1.7 ± 0.1
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	K28/K94[a]	*Triticum urartu* gi|473886714	70	5.3/57.8	3(0)/6	2 ± 0.4/−2 ± 0.7	−1.6 ± 0.2/6.9 ± 0.8	−1.5 ± 0.1/1 ± 0.01	1 ± 0.1/1 ± 0.1

Table 3 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change ± S.E.[i] (SA treated/control) CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Malate dehydrogenase	K61[b]	*Aegilops tauschii* gi|475577109	191	5.3/36.1	3(1)/19	2 ± 0.2/–	4 ± 0.3/–	3.6 ± 0.4/–	1.7 ± 0.03/–
Glyceraldehyde-3-phosphate dehydrogenase	K70/K141[a]	*Aegilops tauschii* gi|475618024	383	6.3/47	5(5)/17	1 ± 0.2/−2.2 ± 0.1	2 ± 0.03/−1.9 ± 0.1	2.2 ± 0.1/1 ± 0.1	1.6 ± 0.1/−5.5 ± 0.1
	K86/K129[a]	*Triticum urartu* gi|473912215	311	6/39.3	5(2)/19	1 ± 0.15/1 ± 0.005	1 ± 0.02/−1.9 ± 0.2	1 ± 0.2/±0.11	1.9 ± 0.1/−3.8 ± 0.05
	K140[c]	*Triticum urartu* gi|473912215	598	6/47.3	10(6)/23	–/1 ± 0.001	–/1 ± 0.1	–/1 ± 0.1	–/−2.3 ± 0.2
	K143[c]	*Aegilops tauschii* gi|475618024	916	6.3/46.9	12(7)/24	–/1 ± 0.2	–/−4.8 ± 0.2	–/1.9 ± 0.02	–/1 ± 0.1
Carbonic anhydrase	K89/K123[a]	*Triticum urartu* gi|474340346	95	7.5/16.3	1(1)/12	1 ± 0.1/1 ± 0.1	1 ± 0.01/1 ± 0.06	1 ± 0.2/1 ± 0.04	3.5 ± 0.1/2.1 ± 0.2
	K114[c]	*Hordeum vulgare* gi|729003	95	8.9/36	1(1)/5	–/−9 ± 0.6	–/1 ± 0.1	–/1 ± 0.01	–/1 ± 0.1
Triosephosphate isomerase	K119[c]	*Secale cereale* gi|1174749	256	5.2/27.1	6(1)/26	–/1 ± 0.2	–/−1.8 ± 0.1	–/1 ± 0.4	–/1 ± 0.1
	K120[c]	*Secale cereale* gi|1174745	176	6/31.9	5(1)/17	–/−1.7 ± 0.2	–/−1.7 ± 0.1	–/−2.5 ± 0.05	–/1 ± 0.2
Sedoheptulose-1,7-bisphosphatase (SBPase)	K138[c]	*Triticum aestivum* gi|300681420	628	6/42.5	14(7)/36	–/1.1 ± 0.03	–/−1.9 ± 0.1	–/−2 ± 0.0003	–/2 ± 0.1
Energy metabolism[k]									
ATP synthase CF1 alpha subunit	K16[b]	*Triticum aestivum* gi|14017569	617	6.2/55.3	9(5)/22	1.5 ± 0.1/–	1 ± 0.3/–	1 ± 0.2/–	1 ± 0.2/–
	K78/K154[a]	*Triticum aestivum* gi|14017569	306	6.1/55.3	6(3)/20	1.5 ± 0.1/1 ± 0.1	1 ± 0.1/−1.7 ± 0.2	1.7 ± 0.1/1 ± 0.1	2.8 ± 0.4/−1.7 ± 0.05
ATP synthase subunit beta, mitochondrial	K24[b]	*Triticum urartu* gi|473798701	503	5.2/57.8	10(5)/26	1.5 ± 0.3/–	1 ± 0.005/–	−1.5 ± 0.1/–	2.2 ± 0.2/–
V-type proton ATPase subunit B2	K75/K100[a]	*Arabidopsis thaliana* gi|15233891	235	5/54	7(1)/20	1.5 ± 0.3/−1.9 ± 0.06	−2 ± 0.8/6.6 ± 0.7	1 ± 0.1/−3.3 ± 0.3	4.5 ± 0.03/1.5 ± 0.1
ATP synthase CF1 beta subunit	K77/K162[a]	*Triticum aestivum* gi|14017579	1198	5.1/53.9	15(9)/44	1.7 ± 0.2/1 ± 0.1	1 ± 0.05/−2.9 ± 0.1	1 ± 0.1/−3.7 ± 0.4	1 ± 0.005/1 ± 0.04
	K90/K153[a]	*Triticum aestivum* gi|14017579	580	5.1/54	9(4)/36	1 ± 0.2/1.7 ± 0.02	3.7 ± 0.4/−7.5 ± 0.4	1 ± 0.05/28.7 ± 0.1	13 ± 0.1/−16 ± 1.1
ATP synthase subunit alpha	K133[c]	*Triticum urartu* gi|474247591	124	5.92/36.6	2(1)/9	–/2 ± 0.01	−/−2.1 ± 0.1	–/1.8 ± 0.1	−/1.1 ± 0.2
Protein synthesis, assembly and degradation[k]									
Harpin binding protein 1	K6[b]	*Triticum aestivum* gi|38679331	187	9.5/29.5	5(1)/20	1 ± 0.1/–	1 ± 0.03/–	1 ± 0.2/–	2.3 ± 0.01/–
Elongation factor Tu, chloroplastic	K14/K144[a]	*Triticum urartu* gi|474198705	1159	4.6/45.8	16(9)/45	4.5 ± 0.1/1 ± 0.04	1 ± 0.05/−2.5 ± 0.3	1 ± 0.04/−2 ± 0.4	1 ± 0.02/1 ± 0.3
Leucine aminopeptidase	K17[b]	*Triticum urartu* gi|474036630	236	5.6/55.8	4(2)/9	2.3 ± 0.2/–	1.7 ± 0.1/–	2 ± 0.2/–	1 ± 0.1/–
70 kDa heat shock protein	K26/K95[a]	*Triticum aestivum* gi|254211611	530	5/73.7	7(4)/13	2 ± 0.2/1 ± 0.2	1.5 ± 0.1/−2 ± 0.1	4.3 ± 0.2/1 ± 0.1	1 ± 0.1/3.6 ± 0.3
	K157[c]	*Triticum urartu* gi|473970552	265	6.2/73.3	7(2)/12	–/1 ± 0.1	–/−3.2 ± 0.2	–/1 ± 0.1	–/2.3 ± 0.5
Glycine-rich RNA-binding protein	K39[b]	*Triticum aestivum* gi|114145394	269	6.3/16	5(2)/42	4.2 ± 0.2/–	−1.9 ± 0.1/–	1 ± 0.3/–	1.6 ± 0.003/–
50S ribosomal protein	K47/K171[a]	*Aegilops tauschii* gi|475532245	108	5.3/21.9	3(0)/19	2.4 ± 0.4/1 ± 0.05	1 ± 0.2/−2.8 ± 0.1	1.9 ± 0.1/1 ± 0.2	1.6 ± 0.03/1 ± 01
20 kDa chaperonin, chloroplastic	K56/K121[a]	*Triticum urartu* gi|474407512	261	6.8/29.8	4(2)/23	1 ± 0.3/1 ± 0.2	1 ± 0.2/−2.6 ± 0.06	−1.9 ± 0.05/1 ± 0.03	1 ± 0.3/−2.1 ± 0.1

(*continued on next page*)

Table 3 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change ± S.E.[i] (SA treated/control)			
						CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Peptidyl-prolyl cis-trans isomerase CYP38	K67/K142[a]	*Triticum urartu* gi|474219338	84	4.8/46.1	4(0)/11	2.5 ± 0.3/1.8 ± 0.003	1 ± 0.2/−1.5 ± 0.04	1 ± 0.1/1 ± 0.01	1.8 ± 0.01/2.5 ± 0.2
RuBisCO large subunit-binding protein	K79[b]	*Triticum urartu* gi|474438538	117	4.9/83.3	3(0)/8	1 ± 0.1/–	−1.6 ± 0.4/–	1 ± 0.2/–	1.5 ± 0.1/–
	K81/K156[a]	*Triticum urartu* gi|474438538	433	7.8/83.3	9(3)/14	1 ± 0.2/2 ± 0.004	−2 ± 0.5/1 ± 0.2	−1.7 ± 0.1/1 ± 0.2	1.8 ± 0.05/1 ± 0.2
Chaperone protein ClpC2	K83/163[a]	*Brachypodium distachyon* gi|357160412	114	6.6/102	3(1)/5	1 ± 0.3/1 ± 0.01	−2 ± 0.3/1 ± 0.2	−1.8 ± 0.2/5 ± 0.02	−2 ± 0.02/1 ± 0.3
Cell division protease ftsH-like protein, chloroplast	K80[b]	*Aegilops tauschii* gi|475605012	526	5.6/71.4	11(4)/21	1 ± 0.2/–	1 ± 0.02/–	1 ± 0.4/–	1.5 ± 0.1/–
Elongation factor G-2, chloroplastic	K160[c]	*Vitis vinifera* gi|359496425	421	5.5/85.5	8(3)/15	–/1 ± 0.01	–/1 ± 0.02	–/1.7 ± 0.005	–/1 ± 0.2
Amino acid metabolism[k]									
5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	K3/K174[a]	*Triticum urartu* gi|473993302	184	5.7/84.8	5(0)/11	2.4 ± 0.2/1.5±	1.5 ± 0.2/1 ± 0.05	1.6 ± 0.1/1.5 ± 0.001	1 ± 0.1/1 ± 0.04
Cysteine synthase	K10/K130[a]	*Triticum urartu* gi|474293105	346	5/98.3	6(4)/9	1 ± 0.1/1 ± 0.04	5.9 ± 0.1/−7 ± 0.3	2.6 ± 0.1/1 ± 0.02	2.4 ± 0.4/1 ± 0.1
	K132[c]	*Triticum urartu* gi|474293105	231	5/98.3	4(3)/7	–/−1.6 ± 0.3	–/1 ± 0.01	–/1 ± 0.001	–/1 ± 0.3
	K13/K137[a]	*Triticum urartu* gi|474315986	230	5.8/35.7	5(1)/18	1.5 ± 0.1/2.9 ± 0.02	−2.4 ± 0.2/−2.3 ± 0.03	−1.7 ± 0.06/1.6 ± 0.04	2.3 ± 0.3/1 ± 0.02
	K62/K170[a]	*Glycine soja* gi|734399710	85	8.1/41.7	2(1)/7	1 ± 0.3/1 ± 0.003	1 ± 0.3/−34.6 ± 1.3	1 ± 0.3/1 ± 0.03	2.7 ± 0.01/1 ± 0.1
	K63[b]	*Glycine soja* gi|734399710	78	8.1/41.7	2(1)/7	1.5 ± 0.2/–	1.5 ± 0.1/–	1 ± 0.3/–	1 ± 0.04/–
Glutamine synthetase	K30/K145[a]	*Triticum aestivum* gi|71362640	403	5.8/47	6(4)/27	1.6 ± 0.1/3.8 ± 0.2	1.5 ± 0.01/1 ± 0.01	1.7 ± 0.2/1.8 ± 0.2	1.8 ± 0.1/1 ± 0.1
	K73/K146[a]	*Triticum aestivum* gi|71362640	355	5.8/47	5(3)/25	1 ± 0.2/1 ± 0.04	1 ± 0.3/−7.7 ± 0.8	1 ± 0.2/−2 ± 0.2	1.5 ± 0.1/−4.3 ± 0.2
	K147[c]	*Hordeum vulgare* gi|121340	143	5.1/47.4	4(1)/15	–/1 ± 0.1	–/1 ± 0.06	–/2.3 ± 0.01	–/1 ± 0.1
Glycine decarboxylase	K33[b]	*Triticum aestivum* gi|22204118	53	5/21.6	2(0)/13	2 ± 0.1/–	2.2 ± 0.05/–	2.1 ± 0.1/–	2.1 ± 0.05/–
GABA transporter 1	K128[c]	*Pyrus* × *bretschneideri* gi|694377037	56	8.9/50.7	1(1)/1	–/1 ± 0.2	–/−1.8 ± 0.07	–/1 ± 0.01	–/6.7 ± 0.1
Defense[k]									
Peroxiredoxin-2E-2	K11[b]	*Triticum urartu* gi|474320948	284	4.5/10.7	3(2)/37	1 ± 0.1/–	−1.6 ± 0.04/–	1 ± 0.2/–	1.5 ± 0.1/–
Single-stranded nucleic acid binding protein	K40/K172[a]	*Triticum aestivum* gi|974605	266	5.2/16.3	5(2)/43	2.4 ± 0.2/−34 ± 0.9	1 ± 0.2/1 ± 0.05	1 ± 0.1/−1.9 ± 0.03	1.8 ± 0.02/1 ± 0.1
Cu/Zn superoxide dismutase	K46/K110[a]	*Triticum aestivum* gi|1572627	247	5.3/20.4	3(2)/43	2.4 ± 0.2/−3.6 ± 0.2	1 ± 0.3/1 ± 0.2	2 ± 0.2/1 ± 0.01	3 ± 0.01/1.54 ± 0.2
Germin-like protein 8–14	K49/K112[a]	*Triticum urartu* gi|473963025	278	5.4/22.1	2(2)/21	1 ± 0.1/1 ± 0.3	−1.5 ± 0.1/6.5 ± 0.1	2.1 ± 0.2/−1.6 ± 0.05	1 ± 0.1/1.8 ± 0.05
2-Cys peroxiredoxin BAS1-like protein	K52/K115[a]	*Morus notabilis* gi|703131051	173	7.7/29.1	2(2)/18	2.9 ± 0.2/1.5 ± 0.3	−1.5 ± 0.6/1 ± 0.3	1 ± 0.2/1 ± 0.1	1 ± 0.004/1 ± 0.2
	K88/K166[a]	*Nicotiana sylvestris* gi|698541875	378	7.6/30	4(2)/24	1 ± 0.3/1.5 ± 0.1	1.6 ± 0.03/−2 ± 0.2	1 ± 0.2/1 ± 0.002	1 ± 0.1/1 ± 0.1
	K91/K136[a]	*Vigna radiata* gi|269980509	50	5.5/28.6	1(1)/5	1 ± 0.1/1.8 ± 0.01	−1.9 ± 0.2/−2 ± 0.03	1 ± 0.1/1 ± 0.1	1 ± 0.1/1 ± 0.2
Ascorbate peroxidase	K57/K122[a]	*Triticum aestivum* gi|226897533	234	5.1/27.9	3(2)/21	1 ± 0.2/1 ± 0.1	1.5 ± 0.01/−2.2 ± 0.1	2.1 ± 0.1/1.9 ± 0.01	1.9 ± 0.01/−1.7 ± 0.1

Table 3 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change ± S.E.[i] (SA treated/control)			
						CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Glutathione *S*-transferase DHAR3	K96[c]	*Brachypodium distachyon* gi|357124703	283	7.7/29.1	5(3)/24	−/1 ± 0.01	−/1 ± 0.3	−/−4.7 ± 0.003	−/2.9 ± 0.3
	K118[c]	*Oryza sativa* Japonica Group gi|1002273449	93	8.3/29.9	2(1)/10	−/1 ± 0.003	−/−8 ± 1.1	−/1 ± 0.1	−/2.5 ± 0.2
Redox signaling[k]									
Thioredoxin M-type	K36[b]	*Triticum aestivum* gi|11135474	54	8.7/19.7	1(0)/9	1 ± 0.2/–	1 ± 0.5/–	1 ± 0.2/–	2.6 ± 0.3/–
	K43/K109[a]	*Triticum aestivum* gi|11135474	206	8.7/19.7	4(2)/23	2.8 ± 0.5/1 ± 0.3	1 ± 0.3/5.8 ± 0.4	1.5 ± 0.3/1 ± 0.02	2 ± 0.1/28.5 ± 0.8
Nucleoside diphosphate kinase 2 (NDPK2)	K41/K107[a]	*Triticum urartu* gi|474449729	285	5.7/26.4	5(3)/20	3.4 ± 0.3/8.4 ± 0.03	1 ± 0.1/1 ± 0.1	−3.9 ± 0.3/1 ± 0.6	1 ± 0.2/3.3 ± 0.4
Thioredoxin H-type 4	K58/K124[a]	*Aegilops tauschii* gi|475610281	242	6.1/33	4(2)/17	1 ± 0.3/1 ± 0.5	1 ± 0.2/1.7 ± 0.06	1.7 ± 0.3/1 ± 0.2	1.8 ± 0.3/−2.3 ± 0.3
	K59/K125[a]	*Aegilops tauschii* gi|475610281	549	6.1/33	10(4)/38	2.4 ± 0.2/1 ± 0.1	1 ± 0.2/1 ± 0.01	1.6 ± 0.5/1.7 ± 0.02	1.8 ± 0.02/1.8 ± 0.1
Cytoskeleton[k]									
Actin	K31/K105[a]	*Glycine* max gi|18532	50	5.2/41.8	1(1)/2	1.5 ± 0.2/1 ± 0.1	2.3 ± 0.4/−7.2 ± 0.6	1.5 ± 0.3/1 ± 0.001	1 ± 0.02/1 ± 0.3
	K50[b]	*Mesostigma viride* gi|5902734	104	5.3/41.8	2(1)/7	2.2 ± 0.1/–	1 ± 0.7/–	−2.6 ± 0.3/–	1 ± 0.02/–
Signal transduction[k]									
Tyrosine phosphorylation protein A	K4/K161[a]	*Triticum aestivum* gi|548319365	105	6.6/74	5(0)/9	1.6 ± 0.1/1 ± 0.1	1.3 ± 0.06/1 ± 0.2	1 ± 0.1/1.6 ± 0.1	3.5 ± 0.01/1 ± 0.1
Ras-related protein Rab7	K35/K104[a]	*Populus euphratica* gi|743840888	51	5.6/23.2	1(1)/3	6.7 ± 0.4/1 ± 0.005	1 ± 0.5/1 ± 0.1	1 ± 0.3/−7.1 ± 0.4	2.5 ± 0.3/85.4 ± 2.3
Translationally-controlled tumor protein	K51[b]	*Triticum aestivum* gi|75246527	253	4.5/18.9	5(3)/30	1.7 ± 0.3/–	1 ± 0.1/–	2 ± 0.2/–	1 ± 0.3/–
Carotenoid accumulation[k]									
Plastid-lipid-associated protein	K15[b]	*Triticum urartu* gi|474126736	436	4.8/34.5	6(4)/28	1 ± 0.3/–	1 ± 0.1/–	2.1 ± 0.3/–	2 ± 0.1/–
	K37/165[a]	*Ostreococcus tauri* gi|693499353	59	10.2/26.6	1(1)/8	6.8 ± 0.5/−5.6 ± 0.5	1 ± 0.3/1 ± 0.6	3.9 ± 0.2/−2.2 ± 0.2	1 ± 0.1/1 ± 0.1
Unknown[k]									
Hypothetical protein F775_17756	K92[c]	*Aegilops tauschii* gi|475516614	72	8.3/35.5	3(0)/8	−/1.9 ± 0.04	−/−6.5 ± 0.9	−/2.1 ± 0.04	−/1 ± 0.2
Unnamed protein product	K151[c]	*Triticum aestivum* gi|669027271	217	5.6/52.6	4(3)/13	−/1 ± 0.1	−/1 ± 0.06	−/1.9 ± 0.04	−/1 ± 0.3
Predicted protein	K168[c]	*Hordeum vulgare* gi|326496957	476	6.3/28	6(4)/44	−/1.7 ± 0.005	−/−2.5 ± 0.2	−/1 ± 0.05	−/1 ± 0.1

[a] Spot no at vegetative stage/spot no at flowering stage.
[b] Spot no at vegetative 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] Petides/% 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 (TW)/control (CW); 75%, 75 + SA (TS1)/75% (CS1); 50%, 50 + SA (TS2)/50%(CS2); RH, RH + SA(TR)/RH(CR).
[j] V/F = fold change at vegetative stage/fold change at flowering stage.
[k] Functional category. N = not detected during flowering stage under severe stress due to early senescence. Different spot ids for same protein at two stages as matching was performed between SA treated and non-treated samples for both stages.

Table 4
List of identified differentially regulated leaf proteins of Lok1 under interactive effect of SA with well watered, water stressed and rehydrated conditions at both stages of development (vegetative and flowering).

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change in Lok1 ± S.E.[i] (SA treated/control)			
						CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Photosynthesis[k]									
NADH dehydrogenase	L2/L114[a]	*Triticum urartu* gi|474097451	86	6.1/73.9	2(1)/5	−1.5 ± 0.2/−9.1 ± 0.7	−1.9 ± 0.1/1 ± 0.04	1 ± 0.1/1 ± 0.1	−2.5 ± 0.2/1 ± 01
RuBisCO small subunit	L20/L91[a]	*Triticum aestivum* gi|132107	301	5.8/13.1	7(1)/41	3.2 ± 0.1/53.9 ± 5.5	1 ± 0.3/−2 ± 0.2	1 ± 0.2/1 ± 0.1	1 ± 0.1/−26 ± 0.01
	L22[b]	*Triticum aestivum* gi|132087	50	8.99/19.7	2(0)/22	3.2 ± 0.03/–	1 ± 0.1/–	1 ± 0.02/–	1 ± 0.01/–
	L33/L95[a]	*Triticum aestivum* gi|11990893	551	9.06/19.8	9(6)/62	−1.9 ± 0.02/−1.6 ± 0.6	5.1 ± 0.2/1 ± 0.05	1 ± 0.01/N	1 ± 0.4/7.05 ± 0.9
	L49/L119[a]	*Triticum aestivum* gi|132107	223	5.8/13	4(3)/30	1 ± 0.1/1.9 ± 0.4	1.8 ± 0.3/1 ± 0.05	1 ± 0.1/N	1 ± 0.1/1 ± 0.1
	L52/L118[a]	*Triticum aestivum* gi|132107	143	5.8/13.3	4(1)/30	1 ± 0.1/−3.6 ± 0.7	1.8 ± 0.04/1 ± 0.0.1	1 ± 0.04/1 ± 0.04	1 ± 0.2/1.5 ± 0.1
	L88[b]	*Triticum aestivum* gi|132107	217	5.8/13.3	5(3)/30	3 ± 0.03/–	6.7 ± 0.4/–	−4.8 ± 0.3/–	1 ± 0.2/–
Thylakoid lumenal 17.4 kDa protein	L28/L122[a]	*Brassica rapa* gi|685372704	90	8.6/25.9	2(0)/11	−1.8 ± 0.2/1.5 ± 0.6	1.9 ± 0.2/1 ± 0.1	1 ± 0.05/1 ± 0.1	1 ± 0.1/1 ± 0.06
Cytochrome b6-f complex iron-sulfur subunit	L29/L96[b]	*Triticum aestivum* UCRIA_WHEAT	51	8.5/24.1	2(1)/12	2.3 ± 0.1/1.5 ± 0.06	3.1 ± 0.2/1 ± 0.1	1 ± 0.1/−13 ± 0.1	1 ± 0.1/−1.8 ± 0.1
	L89[b]	*Triticum aestivum* gi|68566191	265	8.5/24.1	7(2)/33	2.7 ± 0.2/–	−4.2 ± 0.2/–	2.3 ± 0.3/–	−2.5 ± 0.3/–
Oxygen-evolving enhancer protein	L40/L106[a]	*Triticum urartu* gi|474077556	109	8.9/25.7	3(0)/13	1 ± 0.1/1.9 ± 0.3	1.7 ± 0.5/3.5 ± 0.1	1 ± 0.2/1 ± 0.04	1.5 ± 0.4/1 ± 0.2
Chlorophyll a/b binding protein	L44[b]	*Triticum aestivum* gi|302566696	395	5.1/28.7	5(5)/18	1.5 ± 0.05/–	2.8 ± 0.2/–	3.7 ± 0.3/–	2.3 ± 0.2/–
	L45[b]	*Arabidopsis thaliana* gi|16374	111	5.1/25	2(1)/10	1 ± 0.1/–	2.1 ± 0.3/–	1.6 ± 0.04/–	3.6 ± 0.2/–
Ferredoxin-NADP(H) oxidoreductase	L53[b]	*Triticum aestivum* gi|20302473	416	6.9/40.5	8(5)/27	−5 ± 0.6/–	1 ± 0.1/–	−2 ± 0.1/–	1 ± 0.1/–
Photosystem II stability/assembly factor HCF136	L61[b]	*Setaria italica* gi|514768670	537	8.9/43	6(5)/20	−1.7 ± 0.1/–	2.4 ± 0.2/–	2.5 ± 0.3/–	1.8 ± 0.1/–
RUBISCO activase, alpha	L62[b]	*Triticum aestivum* gi|723047999	303	5.3/42.2	4(3)/15	1 ± 0.1/–	1.8 ± 0.6/–	1 ± 0.05/–	1 ± 0.5/–
	L83/L102[a]	*Triticum aestivum* gi|671744544	580	5.6/44.8	12(4)/31	1 ± 0.1/−1.9 ± 0.2	1 ± 0.3/1.2 ± 0.1	1.9 ± 0.02/N	1 ± 0.4/−11 ± 0.1
RuBisCO large subunit	L70/L104[a]	*Triticum aestivum* gi|14017580	334	6.2/53.4	6(3)/15	1 ± 0.1/−2.1 ± 0.3	3 ± 0.2/1 ± 0.1	1.5 ± 0.3/N	3.4 ± 0.1/−2.4 ± 0.3
	L72/L134[a]	*Triticum aestivum* gi|14017580	954	6.2/53	15(9)/37	1 ± 0.1/3.6 ± 0.2	2 ± 0.1/1 ± 0.01	1 ± 0.1/N	1 ± 0.4/1 ± 0.1
	L135[c]	*Triticum aestivum* gi|14017580	334	6.2/53.4	6(3)/15	–/−1.7 ± 0.03	–/1 ± 0.05	–/1 ± 0.04	–/1 ± 0.01
Photosystem I subunit VII	L94[c]	*Oryza sativa* Japonica Group gi|11466848	621	6.5/9.4	9(7)/91	–/4.1 ± 0.7	–/1 ± 0.1	–/N	–/15.3 ± 2.0
Thylakoid lumenal 17.4 kDa protein	L28/L122[a]	*Brassica rapa* gi|685372704	90	8.5/25.9	2(0)/11	−1.8 ± 0.2/1.5 ± 0.06	1.9 ± 0.2/1 ± 0.1	1 ± 0.05/1 ± 0.1	1 ± 0.1/1 ± 0.06
Thylakoid lumenal 15 kDa protein	L120[c]	*Nicotiana tomentosiformis* gi|697098258	184	5.4/23.6	1(1)/11	–/2.2 ± 0.5	–/1 ± 0.1	–/1 ± 0.03	–/1 ± 0.1

Table 4 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change in Lok1 ± S.E.[i] (SA treated/control) CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Carbon metabolism[k]									
Transketolase	L1[b]	*Triticum urartu* gi|474352176	532	5.4/74	13(3)/31	−2 ± 0.1/–	1.7 ± 0.1/–	1 ± 0.1/–	2.1 ± 0.5/–
	L15/L136[a]	*Triticum urartu* gi|474352176	707	5.4/68.9	13(5)/28	1 ± 0.4/1.5 ± 0.1	1.7 ± 0.2/1 ± 0.06	1.5 ± 0.1/N	2.3 ± 0.2/1 ± 0.2
	L79[b]	*Ricinus communis* gi|255541252	83	6.52/81.6	3(1)/7	1 ± 0.1/–	1.6 ± 0.1/–	1.6 ± 0.1/–	1.7 ± 0.3/–
	L80[b]	*Aegilops tauschii* gi|475481099	425	5.4/69.4	12(4)/26	1 ± 0.2/–	1.6 ± 0.2/–	1 ± 0.1/–	1.6 ± 0.03/–
6-phosphogluconate dehydrogenase, decarboxylating	L4[b]	*Setaria italica* gi|514786876	240	5.6/52.9	6(2)/15	−2.6 ± 0.2/–	1.9 ± 0.5/–	1 ± 0.1/–	1 ± 0.1/–
	L115[c]	*Bathycoccus prasinos* gi|612395739	159	5.6/56.9	4(1)/8	–/2.1 ± 0.1	–/1.7 ± 0.1	–/1.6 ± 0.05	–/1.7 ± 0.2
Phosphoglycerate kinase	L9[b]	*Triticum aestivum* gi|129915	271	6.6/49.9	5(2)/13	−1.6 ± 0.2/–	−1.6 ± 0.2/–	−2 ± 0.2/–	−1.5 ± 0.2/–
	L11[b]	*Solanum tuberosum* gi|82621134	56	8.2/50.3	2(0)/5	1 ± 0.2/–	−2.2 ± 0.2/–	−1.9 ± 0.6/–	1 ± 0.2/–
Fructose-bisphosphate aldolase (FBPase), chloroplastic	L10[b]	*Triticum aestivum* gi|223018643	473	5.9/42.2	9(5)/28	1 ± 0.1/–	4.2 ± 0.1/–	1 ± 0.1/–	1 ± 0.04/–
	L56/L113[a]	*Brachypodium distachyon* gi|357157399	565	6.3/42.2	7(6)/20	1 ± 0.2/−3.3 ± 0.3	1.6 ± 0.1/1 ± 0.1	1 ± 0.1/1	1 ± 0.06/1 ± 0.03
	L59/L128[a]	*Triticum aestivum* gi|223018643	302	5.9/42.2	7(5)/25	1 ± 0.1/−1.7 ± 0.3	1.6 ± 0.4/1 ± 0.1	1 ± 0.1./N	1 ± 0.3/1
	L60[b]	*Setaria italica* gi|514807943	335	5.9/37.3	5(4)/15	1 ± 0.2/–	2.7 ± 0.2/–	1 ± 0.5/–	1.8 ± 0.6/–
	L132[c]	*Triticum urartu* gi|473936969	1191	8.5/69.9	12(10)/23	–/2.3 ± 0.04	–/−3.4 ± 0.3	–/1	–/1 ± 0.03
Phosphoglycolate phosphatase	L13/L107[a]	*Aegilops tauschii* gi|475589243	432	4.9/33.6	6(5)/18	1 ± 0.02/−1.6 ± 0.1	1.5 ± 0.4/1 ± 0.3	1 ± 0.1/1 ± 0.03	1 ± 0.2/1 ± 0.02
	L47[b]	*Setaria italica* gi|514802286	193	5.9/39.6	3(3)/7	1 ± 0.3/–	2.8 ± 0.2/–	1 ± 0.06/–	2.3 ± 0.2/–
	L111[c]	*Musa acuminata* gi|695053889	78	7.6/40.3	1(1)/3	–/1 ± 0.2	–/−7.9 ± 0.3	–/1 ± 0.1	–/1
Carbonic anhydrase	L38/L125[a]	*Hordeum vulgare* gi|729003	374	8.9/35.7	5(4)/20	1 ± 0.05/−4.8 ± 0.4	1.7 ± 0.4/1 ± 0.1	1 ± 0.2/1	1.5 ± 0.3/1 ± 0.03
	L39[b]	*Hordeum vulgare* gi|729003	108	8.9/35.7	2(1)/8	1.5 ± 0.1/–	1.8 ± 0.05/–	2.1 ± 0.05/–	1 ± 0.2/–
	L46[b]	*Hordeum vulgare* gi|729003	102	8.9/35.7	2(1)/8	1.5 ± 0.1/–	2 ± 0.06/–	4 ± 0.4/–	2 ± 0.5/–
Malate dehydrogenase	L55/L129[a]	*Aegilops tauschii* gi|475577109	244	5.3/36.1	3(3)/11	1.5 ± 0.05/1.7 ± 0.2	1.6 ± 0.3/1 ± 0.1	1 ± 0.2/1 ± 0.1	1 ± 0.1/1 ± 0.04
	L58[b]	*Triticum aestivum* gi|49343245	341	5.7/35.8	5(3)/27	1.8 ± 0.02/–	2.2 ± 0.04/–	1 ± 0.4/–	1 ± 0.2/–
Triosephosphate isomerase	L42[b]	*Secale cereale* gi|475578260	425	6/31.9	7(5)/24	1.5 ± 0.2/–	1.8 ± 0.1/–	2.1 ± 0.1/–	1 ± 0.1/–
	L43[b]	*Triticum aestivum* gi|11124572	391	5.4/27	7(4)/35	−2 ± 0.01/–	2.4 ± 0.2/–	1.7 ± 0.1/–	1.6 ± 0.1/–
Sedoheptulose-1,7-bisphosphatase (SBPase)	L65/L99[a]	*Triticum aestivum* gi|300681420	384	6.2/42.5	6(4)/21	1.7 ± 0.3/11 ± 0.2	1.8 ± 0.5/3 ± 0.3	4.4 ± 0.3/1 ± 0.1	1.6 ± 0.1/2.1 ± 0.1
UTP–glucose-1-phosphate uridylyltransferase (UGPase)	L73[a]	*Hordeum vulgare* gi|6136111	778	5.2/51.8	10(8)/23	1 ± 0.2/–	1 ± 0.6/–	1 ± 0.1/–	1.6 ± 0.2/–
Glyceraldehyde-3-phosphate dehydrogenase	L66[b]	*Aegilops tauschii* gi|475618024	839	6.3/46.9	10(8)/24	1 ± 0.3/–	1.7 ± 0.5/–	1 ± 0.4/–	1.8 ± 0.1/–

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Table 4 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change in Lok1 ± S.E.[i] (SA treated/control)			
						CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
	L67[b]	*Triticum urartu* gi|474305966	928	7.6/39.3	11(7)/34	1 ± 0.1/–	1.6 ± 0.4/–	1 ± 0.1/–	1.5 ± 0.4/–
	L86[b]	*Triticum urartu* gi|474305966	298	6/39.3	5(4)/13	1 ± 0.2/–	2.2 ± 0.6/–	1 ± 0.2/–	1 ± 0.02/–
Energy metabolism[k]									
ATP synthase beta subunit	L7/L93[b]	*Triticum urartu* gi|474124791	82	5.1/50.6	1(1)/2	1 ± 0.2/2.7 ± 0.5	2.5 ± 0.5/−4.2 ± 0.3	1 ± 0.3/2.3 ± 0.3	1 ± 0.2/−2.5 ± 0.1
ATP synthase subunit	L12[a]	*Triticum aestivum* gi|285014508	222	8.2/40	5(2)/14	1 ± 0.1/–	1 ± 0.06/–	1.5 ± 0.05/–	1 ± 0.1/–
ATP synthase delta chain, chloroplast	L34[b]	*Aegilops tauschii* gi|475627717	637	4.5/17.7	9(6)/55	1 ± 0.3/–	3 ± 0.2/–	1.8 ± 0.5/–	1.5 ± 0.1/–
ATP synthase CF1 beta subunit	L74[b]	*Triticum aestivum* gi|14017579	1334	5.1/53.9	20(10)/52	1 ± 0.1/–	29.1 ± 1.3/–	2.7 ± 0.04/–	2.4 ± 0.2/–
ATP synthase beta subunit	L105[c]	*Triticum aestivum* gi|525291	106	5.6/59.3	3(1)/7	–/−31 ± 1.0	–/−10 ± 0.8	–/−7 ± 0.8	–/1 ± 0.1
Protein synthesis, assembly and degradation[k]									
ATP-dependent Clp protease ATP-binding subunit clpA-like protein CD4B	L3[b]	*Triticum urartu* gi|473787307	116	5.9/98.4	6(0)/10	−1.7 ± 0.4/–	10.7 ± 1.1/–	1 ± 0.1/–	1 ± 0.1/–
Heat shock protein 70	L16[b]	*Populus trichocarpa* gi|566191989	426	5.1/71.5	10(4)/18	−1.9 ± 0.2/–	1.6 ± 0.1/–	1.6 ± 0.4/–	1 ± 0.05/–
	L78[b]	*Triticum urartu* gi|474389043	565	5.3/77.3	16(4)/24	1 ± 0.2/–	29.2 ± 1.8/–	2.7 ± 0.2/–	2.4 ± 0.4/–
	L84[b]	*Triticum urartu* gi|473970552	520	6.2/76.3	7(6)/14	1 ± 0.1/–	1.6 ± 0.3/–	1 ± 0.3/–	1.6 ± 0.1/–
RuBisCO large subunit-binding protein	L18/L92[a]	*Triticum aestivum* gi|134102	1142	4.8/57.6	16(11)/35	1 ± 0.3/3.2 ± 0.4	2 ± 0.2/6.7 ± 0.4	1 ± 0.3/−4.8 ± 0.2	3.2 ± 0.2/1 ± 0.1
	L75/L108[a]	*Triticum urartu* gi|474438538	433	7.8/83.3	9(3)/14	−3 ± 0.1/1.6 ± 0.1	1 ± 0.2/1 ± 0.3	1 ± 0.2/1 ± 0.1	1.9 ± 0.1/1 ± 0.1
	L77[b]	*Aegilops tauschii* gi|475569550	1172	5/60.9	17(11)/35	1 ± 0.2/–	1.8 ± 0.4/–	1.6 ± 0.1/–	1 ± 0.2/–
Glycine-rich RNA-binding protein	L23[b]	*Aegilops tauschii* gi|475522294	179	5.4/12.3	4(2)/34	3.4 ± 0.03/–	1.9 ± 0.2/–	5.6 ± 0.1/–	1.7 ± 0.2/–
50S ribosomal protein	L27[b]	*Triticum urartu* gi|474267217	83	7.9/17.7	2(0)/14	1 ± 0.2/–	1.7 ± 0.2/–	2.6 ± 0.04/–	2 ± 0.2/–
Pyridoxal biosynthesis protein PDX1.1-like	L50/L127[a]	*Oryza brachyantha* gi|573950163	174	5.9/33.7	1(1)/20	1 ± 0.3/1.8 ± 0.2	1 ± 0.3/1 ± 0.03	2.6 ± 0.1/1 ± 0.04	1 ± 0.1/1 ± 0.04
Chloroplast stem-loop binding protein of 41 kDa	L63[b]	*Brachypodium distachyon* gi|357111159	319	7.1/41.4	4(3)/9	1 ± 0.3/–	2.8 ± 0.1/–	1 ± 0.1/–	1.6 ± 0.1/–
Peptidyl-prolyl cis-trans isomerase CYP38	L69[b]	*Aegilops tauschii* gi|475622947	84	4.7/44.5	4(0)/11	1 ± 0.2/–	1.9 ± 0.1/–	1 ± 0.2/–	2.4 ± 0.2/–
Chaperone protein ClpC1	L82[b]	*Oryza sativa* Japonica Group gi|347602486	67	6.1/102	3(0)/4	1 ± 0.2/–	2.2 ± 0.3/–	1.7 ± 0.1/–	1 ± 0.1/–
Elongation factor G-2	L87[b]	*Brachypodium distachyon* gi|357164996	332	4.9/84.3	7(4)/13	53.9 ± 2.2/–	1 ± 0.03/–	1.9 ± 0.1/–	1 ± 0.3/–
Elongation factor Tu	L117[c]	*Triticum urartu* gi|474198705	547	4.6/45.8	9(6)/30	–/−2 ± 0.1	–/1 ± 0.1	–/N	–/1 ± 0.02
	L133[c]	*Triticum urartu* gi|474198705	473	4.6/45.8	7(4)/26	–/−1.6 ± 0.2	–/1 ± 0.1	–/1 ± 0.03	–/1 ± 0.1
30S ribosomal protein S5	L130[c]	*Zea mays* gi|226492493	95	5.2/34.2	3(0)/10	–/−14 ± 1.0	–/1 ± 0.02	–/1 ± 0.1	–/1 ± 0.04
Amino acid metabolism[k]									
S-adenosylmethionine synthase	L5[b]	*Triticum urartu* gi|474105890	447	5.6/43.2	8(4)/20	1.5 ± 0.3/–	1.5 ± 0.2/–	1.9 ± 0.1/–	1 ± 0.2/–
Glutamate decarboxylase	L6[b]	*Triticum aestivum* gi|300681536	52	5.5/54.5	2(0)/6	1.6 ± 0.4/–	1 ± 0.1/–	1 ± 0.2/–	1 ± 0.04/–

Table 4 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change in Lok1 ± S.E.[i] (SA treated/control) CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Glutamine synthetase	L14/L100[b]	*Triticum aestivum* gi|251832986	370	5.75/47.4	6(3)/27	1 ± 0.05/1 ± 0.07	2 ± 0.5/1.8 ± 0.3	2 ± 0.1/−5.7 ± 0.6	1 ± 0.1/1 ± 0.1
	L68/L101[a]	*Triticum aestivum* gi|71362640	299	5.7/47.4	8(2)/25	1 ± 0.3/1 ± 0.1	1.5 ± 0.2/3.5 ± 0.3	1 ± 0.03/1 ± 0.03	1.5 ± 0.1/1.7 ± 0.1
	L103[c]	*Triticum aestivum* gi|71362640	241	5.7/47.4	8(1)/25	–/1 ± 0.05	–/1 ± 0.1	–/1 ± 0.1	–/1.7 ± 0.07
GABA	L51/L110[b]	*Prunus mume* gi|645217729	56	8.9/50.7	1(1)/1	−3.2 ± 0.2/1 ± 0.1	3.2 ± 0.4/−6.4 ± 0.4	1.8 ± 0.2/N	1 ± 0.06/1 ± 0.2
Cysteine synthase	L54/L109[b]	*Aegilops tauschii* gi|475604383	230	5.8/34.2	5(1)/18	−2.2 ± 0.2/−2.2 ± 0.3	3.2 ± 0.3/−3.6 ± 0.5	1.8 ± 0.2/N	1 ± 0.2/1 ± 0.05
	L57[b]	*Zea mays* gi|2829688	66	5.9/34.3	2(0)/8	1 ± 0.1/–	2.1 ± 0.05/–	1 ± 0.1/–	1 ± 0.03/–
Ketol-acid reductoisomerase,	L76/L116[b]	*Nelumbo nucifera* gi|357132462	83	6.8/64.4	2(1)/14	1 ± 0.1/3.2 ± 0.1	2 ± 0.5/1 ± 0.2	1 ± 0.3/N	1 ± 0.2/1 ± 0.1
5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	L81[b]	*Triticum urartu* gi|473993302	150	5.7/84.4	4(1)/9	1 ± 0.2/–	1.9 ± 0.5/–	1 ± 0.3/–	1.7 ± 0.08/–
Defense[k]									
Late embryogenesis abundant protein D-29	L25[b]	*Glycine soja* gi|734319188	93	7.1/48.3	48/64	1 ± 0.1/–	2 ± 0.1/–	2.7 ± 0.2/–	2 ± 0.09/–
Germin-like protein 8–14	L31/L97[a]	*Triticum urartu* gi|473963025	198	5.4/22.1	2(2)/21	1 ± 0.1/5.3 ± 0.4	3 ± 0.4/1 ± 0.1	1.8 ± 0.3/1 ± 0.1	1.5 ± 0.08/1 ± 0.2
	L32[b]	*Triticum urartu* gi|473963025	278	5.4/22.1	2(2)/21	2.3 ± 0.2/–	3.1 ± 0.5/–	1 ± 0.1/–	1 ± 0.03/–
2-Cys peroxiredoxin BAS1	L35[b]	*Triticum aestivum* gi|2829687	147	5.7/23.4	2(1)/17	10.2/–	1.7 ± 0.05/–	1 ± 0.1/–	1.5 ± 0.2/–
	L36[b]	*Morus notabilis* gi|703131051	173	7.7/29.1	2(2)/18	10 ± 0.04/–	3.5 ± 0.6/–	1 ± 0.02/–	1 ± 0.1/–
	L37[b]	*Malus domestica* gi|657999067	61	7.7/29.5	1(1)/8	1 ± 0.06/–	1.7 ± 0.2/–	1 ± 0.1/–	1.5 ± 0.2/–
	L90/L131[a]	*Glycine soja* gi|734316698	50	4.53/19.8	1(1)/7	4 ± 0.3/−1.6 ± 0.2	1 ± 0.3/1 ± 0.1	1/N	15 ± 0.6/1 ± 0.05
Glutathione *S*-transferase DHAR3,	L41[b]	*Triticum urartu* gi|474299547	262	6/28.6	5(2)/22	−2 ± 0.1/–	1.6 ± 0.1/–	1.5 ± 0.1/–	2.9 ± 0.1/–
Triticain alpha	L71[b]	*Triticum aestivum* gi|111073715	108	5/51.6	2(1)/6	−3 ± 0.1/–	1 ± 0.1/–	1 ± 0.3/–	1.9 ± 0.1/–
Redox signaling[k]									
Thioredoxin M-type	L24/L121[a]	*Triticum aestivum* gi|11135474	54	8.7/19.7	1(0)/9	1 ± 0.1/1.7 ± 0.3	−29.8 ± 1.3/1 ± 0.1	1 ± 0.2/N	1 ± 0.2/1 ± 0.04
	L123[c]	*Triticum urartu* gi|474434045	206	5.3/12.5	4(2)/37	–/1.5 ± 0.1	–/1 ± 0.01	–/1 ± 0.06	–/1 ± 0.02
Thioredoxin H type4	L48/L98[a]	*Aegilops tauschii* gi|475610281	259	6.1/33	7(3)/28	1 ± 0.3/11 ± 1.0	1.8 ± 0.5/1 ± 0.05	2 ± 0.1/N	2 ± 0.1/1.5 ± 0.1
Nucleoside diphosphate kinase 2	L85[b]	*Triticum urartu* gi|474449729	192	5.7/23.6	3(1)/11	1 ± 0.1/–	1 ± 0.1/–	2.8 ± 0.2/–	1 ± 0.1/–
Cytoskeleton[k]									
Actin	L19/L112[a]	*Cicer arietinum* gi|502102666	380	5.23/41.9	7(4)/30	1 ± 0.03/2.8 ± 0.1	1 ± 0.1/1 ± 0.1	2.4 ± 0.1/N	5.6 ± 0.5/1 ± 0.03
	L124[c]	*Mesostigma viride* gi|5902734	104	5.3/41.8	2(1)/7	–/2 ± 0.2	–/1 ± 0.1	–/1 ± 0.05	–/1 ± 0.04
Signal transduction[k]									
Translationally-controlled tumor protein	L30[b]	*Cucumis melo* gi|661902842	68	4.5/18.8	2(1)/11	−1.9 ± 0.03/–	5.1 ± 0.6/–	2.7 ± 0.01/–	1 ± 0.1/–
Unknown[k]									
Unnamed protein product	L8[b]	*Triticum aestivum* gi|669027271	55	5.6/52.6	2(0)/7	1 ± 0.1/–	1.9 ± 0.3/–	1.6 ± 0.1/–	1 ± 0.1/–

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Table 4 (*continued*)

Protein	Spot no[d]	Species accession no[e]	Score[f]	pI/MW[g]	Petides/% Seq coverage[h]	Fold change in Lok1 ± S.E.[i] (SA treated/control) CON (V/F)[j]	75% (V/F)[j]	50% (V/F)[j]	RH (V/F)[j]
Predicted protein	L126[c]	*Hordeum vulgare* gi|326523629	319	7.1/41.4	4(3)/9	−/− 1.6 ± 0.3	−/1 ± 0.05	−/1 ± 0.1	−/1 ± 0.02

[a] Spot no at vegetative stage/spot no at flowering stage.
[b] Spot no at vegetative 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] Petides/% 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 (TW)/control (CW); 75%, 75 + SA (TS1)/75% (CS1); 50%, 50 + SA (TS2)/50%(CS2); RH, RH + SA(TR)/RH(CR).
[j] V/F = fold change at vegetative stage/fold change at flowering stage.
[k] Functional category. N = not detected during flowering stage under severe stress due to early senescence. Different spot ids for same protein at two stages as matching was performed between SA treated and non-treated samples for both stages.

system in SA conferred drought tolerance. SA enhanced activities of SOD, CAT, APX and GR in both varieties at vegetative stage providing defense against drought (Supplementary Fig. S4). The enzymatic activities increased with increasing intensity of water stress under SA as highest activity observed in TS2 then in TS1 and TR. SA showed major influence on antioxidant system of Lok1 as compared to Kundan. Increased abundance of antioxidative enzymes like SOD (spot K46), APX (spots K57, K122) and GST (spots K96, L41) and GLP (spots K49, L31, L32) under SA indicated stress acclimation through redox homeostasis. Patel and Hemantaranjan [78] reported increase in antioxidative enzyme activity and antioxidants level as a selection criteria for drought tolerance in chickpea under the effect of SA. The increased levels of antioxidants glutathione (GSH) and ascorbate (AsA) in SA treated Lok1 and Kundan plants (Supplementary Figs. S5–S6) alleviated the detrimental effects of drought stress in wheat as reported elsewhere [28]. Sulfur metabolism is reported to be related to glutathione generation and there was SA induced expression of CS (spots K10, K137, L54, L109, Clusters 2–3, 7–8) which maintained glutathione pool under stress (TS1 and TS2) and rehydration (TR) [79,80]. PCA biplot at both stages showed the predominant role of proline in SA induced drought tolerance under stress and rehydration for Kundan than Lok1 (Fig. 1 & Supplementary Fig. S7A). Abundance of glutamate pathway proteins GDC (spot K33), GS (spots K30, L14), GABA transporter (spot L51) and GltDC (spot L6) accounted for proline accumulation contributing towards osmoprotection [81]. Western blot analysis probed with antibody against GS validated the expression pattern under SA treatment in water stressed and rehydrated plants (Supplementary Figs. S19–S20) The inverse relation of MDA equivalents with SA demonstrated the membrane protection in TS1, TS2 and TR plants. The role of thioredoxin (spots K43, K58, L48) and Cys-Prx (spots K52, K88, K91, L35, L37, L90) in membrane stability and redox homeostasis supported their abundance under SA conferring drought tolerance [82,83]. Merewitz et al. [84] reported the role of methionine metabolism SAMS (spot L5) and SHAMS (spots K3, K174, L81) in SA induced early response to drought. The risk of protein misfolding increases during stress but SA enhanced expression of Hsps (spots K26, K95, L78), chaperons (spots K163, L82), RBP (spots K75, K79, K81, K156, L18, L92) and PPI (spots K67, K142, L69) in stress and rehydration. The expression of these proteins (Clusters 1–4 and 5–7) was more pronounced in stressed Lok1 plants under SA although they were also found in Kundan. SA induced stress acclimation enhanced protein turnover through eukaryotic TF-G (spots K14, K160, L87), 50S RP and 30S RP (spots K39, K47, L23, L27) [66,85]. Increased oxidative stress due to ROS production has also been reported in plants undergoing senescence [86]. Tolerant plants of TS1 and TS2 at flowering stage maintained the redox balance through abundance of APX (spot K122), GLP (spot K112) (Cluster 6) and increased level of antioxidants protecting plants from early senescence. Decreased abundance of some proteins (spots K118, K136, K166, K172) related to redox balance in Kundan suggested the influence of SA on resource reallocation from source to sink under yield limiting drought stress. SA showed negative influence of SA on major antioxidative enzymes activity during flowering stage predominantly in Lok1 supported by the decreased abundance of defense and redox balance related proteins (spots L121, 123, L131). During anthesis, unlike other enzymes the activity of catalase showed pronounced increase on SA treatment (Supplementary Fig. S4C1) and showed maximum variability in PC1 (Fig. 1). Increased catalase activity during grain filling in chickpea has been reported under water stress and SA treatment to detoxify the excess H_2O_2 [77] which supports our findings.

4.4. Redox and SA signaling

The interplay between SA and redox signaling highlighted by some SA transcriptional regulators, i.e., Non-expressor of pathogenesis related protein (NPR1) and TGACG sequence-specific binding proteins (TGAs) functioning as redox sensors. These redox sensors are directly or indirectly regulated by some Thioredoxin (TRX)/Glutaredoxin (GRX) oxidoreductase enzymes [87,88,89]. NPR1, in monomer form, is required downstream of SA to interact with TGAs in nucleus to activate many defense genes [87,90]. Monomerization of the NPR1 protein to the active form is triggered by thioredoxin to be translocated into nucleus for downstream processing [87]. Trx (M and H type; spots K43, K58, K109, K125, L48) abundance demonstrated the interplay between redox and SA signaling activating different defense responses. Trxs activated target enzymes (PRK, RA, SBPase, ATPase, GAPDH) involved in metabolism [91]. Abundance of redox signaling protein NDPK2 has been reported under SA application depicting its role in defense with SA mediated defense responses [92]. NDPK2 (spots K41, K107, L85; Clusters 2, 3 and 8) expression was induced by SA under stress (TS1) and recovery (TR) for ROS detoxification as reported by [93].

4.5. Cytoskeleton and signal transduction

Drought tolerance level is also decided by actin protein which is reported to control drought-induced intracellular chloroplast positioning in mesophyll under interaction with SA as studied in barley [94]. We found abundant actin protein (spots K31, L19; Clusters 2–3). Kim et al. [95] reported function of TCTP in ABA induced stomatal closure under water stress to enhance drought tolerance. In our study, TCTP (spots

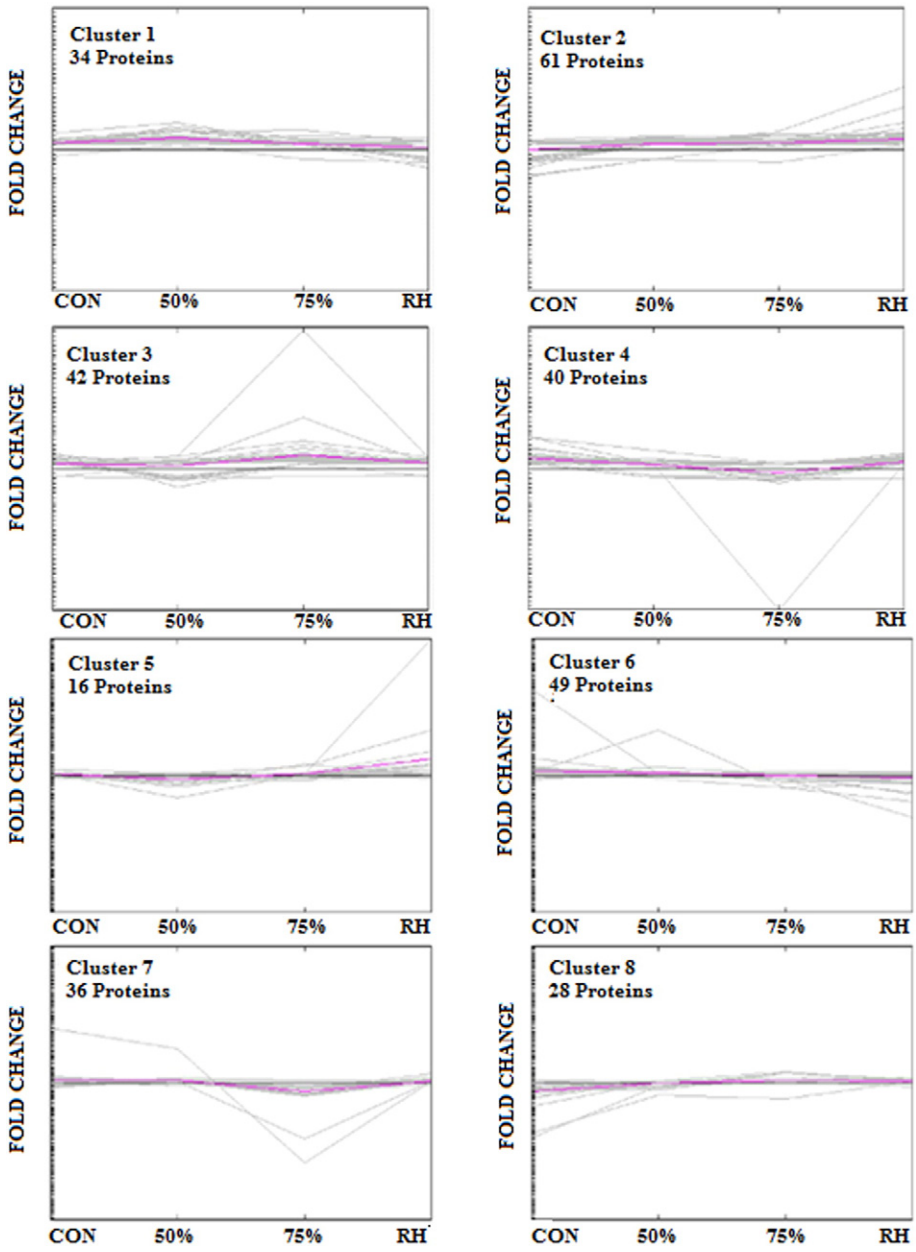

Fig. 5. k mean clustering of identified differentially expressed proteins of Kundan and Lok1 wheat varieties under interactive effect of SA and drought at vegetative and flowering stage. Clusters I–IV represents protein clusters at vegetative stage and Clusters V–VIII represents protein clusters at flowering stage. Protein clustered on the basis of their expression pattern under SA.

K51, L30; Cluster 1) was found to be accumulated under the influence of SA conferring enhanced drought tolerance.

4.6. Drought responsive proteins specific to tolerant and sensitive variety

SA positively influenced metabolism and stress signaling in tolerant Kundan variety further enhancing its tolerance capability. PRK increased the activity of RuBisCO and PPDK1 (spot K5; Cluster 1) regulated the glycolytic enzymes [96] in Kundan showing stress acclimation. Stress signaling proteins like Tyrosine Phosphorylation protein A (spots K4, K161; Clusters 2 and 6) regulating oxidative stress signaling pathway under drought [97] and ras related GTPase (spots K35, K104; Clusters 4–5) activated by TCTP involved in signal cascade for stomatal closure were accumulated specifically in Kundan mostly on stress recovery (TR) and some in TS2. Carotenoids functions in stabilization of lipid membranes, light harvesting for photosynthesis and protecting the photosystem from photo-oxidation [98]. Interactive effect of water stress (TS2) and SA caused increased expression of Putative plastid-lipid-associated protein 13 (spots K37; Cluster 4) in tolerant variety. Leucine aminopeptidase (spot K17; Cluster 1) involved in protein catabolism enhanced by SA providing amino acid for osmoregulation contributed towards drought tolerance in Kundan as also reported by Cheng et al. [56].

The sensitivity of Lok1 towards water stress was higher causing the increase in defense, energy production and protective responses against stress under SA. LEA protein (spot L25; Cluster 1) abundance in TS1, TS2 and TR protected membrane and other functional proteins from oxidative stress under drought [66,65]. The abundance of pyridoxine biosynthesis protein (spots L50, L127; Clusters 3 and 6) explained the increase in defense against drought under SA (TS1) as reported by Gharechahi et al. [84]. Chloroplast stem loop binding protein (spot L63; Cluster 3) maintains chloroplast integrity a defense response against water

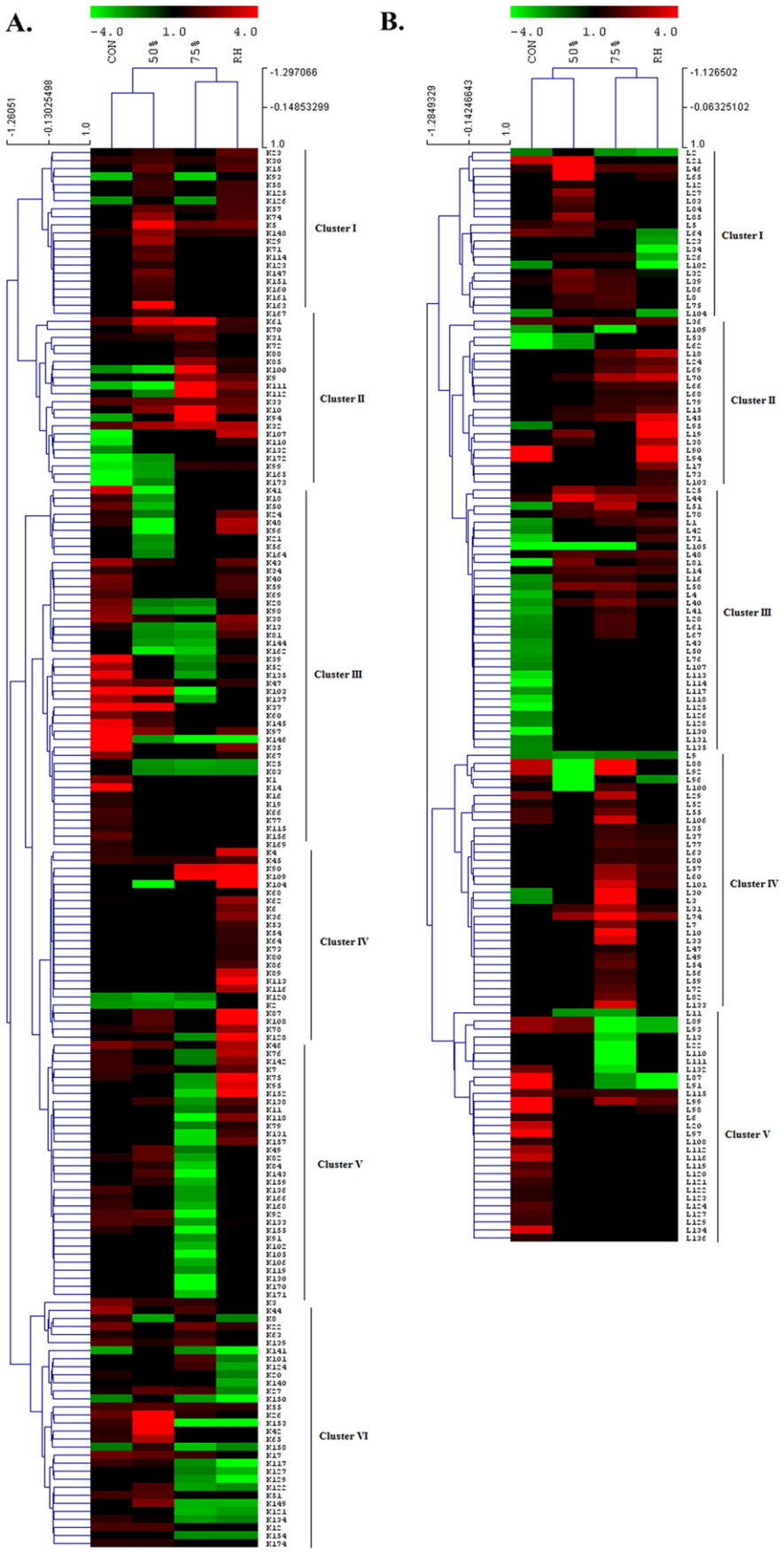

Fig. 6. Heat map showing expression pattern and hierarchical clustering of identified proteins during both growth stages of A. Kundan; B. Lok1 with SA treatment under well watered (control), stressed (50% and 75%) and rehydration (RH) condition. Expression value represents ratio of SA treated and their respective control. Red colour indicates a positive abundance in protein spots; green colour denotes a negative abundance in protein spots.

stress in SA treated Lok1 (TS1) and Ketol acid reductoisomerase (spots L76, L116; Cluster 3 and 6) involved in branched chain amino acid synthesis conferred drought tolerance [74].

4.7. Yield

Wheat Yield is a function of seed number and weight; both largely controlled by seed sink strength [99]. In our study, seed number and seed weight per plant were increased under SA treatment in both varieties (Fig. 2). SA positively impacted wheat yield under water deficit by primarily increasing grain number and decrease in 1000 seed weight had no direct effect on yield. Earlier reports also suggest the importance of grain number in yield of wheat [100] and negative indirect effect of grain number per plant on 1000 seed weight [101]. Aldesuquy et al. [102] and Sharafizad et al. [103] have reported the enhanced productivity of wheat under drought through positive influence of SA on yield components. SA improved the yield potential under limiting conditions through resource reallocation to developing sink [102,104]. Abundance of TK (spots K93, K158, L136), SBPase (spot L99) and UGPase (spot K101) in SA treated plants under stress (TS1 and TS2; Clusters 6–7) correlated with the accumulation of sugars in source to be translocated towards sink for starch biosynthesis [105,106]. PGD (spots K148, L115; Clusters 6–7) abundance at anthesis explained its role in starch biosynthesis, regeneration of NADPH for nitrate assimilation and glutamine synthesis in leaves for fulfilling starch and N requirement by developing seeds [107]. This was corroborated by increased seed starch content and seed weight under SA (Fig. 2B, Supplementary Fig. S2). The abundance of GS (spots K145, K147, L101) and GABA transporter (spot K128) in wheat leaves under SA (Clusters 5–6) explained the role of SA in increasing sink potential by providing glutamine serving as a reservoir of nitrogen for seed development at both stress and recovery [108]. The resource reallocation from source to developing sink during grain filling explained the alleviation of yield limitation by SA under drought in both sensitive and tolerant variety.

5. Conclusion

SA (0.5 Mm) treatment significantly increased the growth and drought tolerance in two winter wheat varieties: drought-tolerant Kundan and drought-sensitive Lok1. These responses were manifested by increased biomass accumulation, antioxidative enzymes activity, antioxidants, osmolytes (proline and soluble sugars), A_N, g_s, WUE and chlorophyll. SA positively influenced these traits in both varieties with pronounced positive effect on Kundan. Lok1 under SA had increased level of tolerance at par with the untreated Kundan. SA induced tolerance in Kundan was evident even under severe stress but in Lok1 it was higher in moderate stress. Severe stress caused early senescence under interactive effect of stress with SA in Lok1 as a drought avoidance mechanism. Proteomics also revealed the ameliorating effects of SA under water stress more in Kundan than in Lok1. At vegetative stage, proteins involved in photosynthesis, carbon metabolism, energy metabolism, amino acid and protein metabolism, defense and redox signaling were majorly involved in SA induced drought tolerance in both varieties. In tolerant variety, SA positively regulated proteins related to carotenoid accumulation and signal transduction for drought tolerance. SA enhanced the inherit tolerance potential of Kundan by increased physiological functions, carbon metabolism, redox homeostasis, osmoprotection and protein turnover. While the shift towards defense, osmoprotection, protein metabolism for maintaining protein turnover and energy metabolism by SA for stress acclimation was observed in Lok1. Yield limitation by water stress was relieved by SA with increased yield parameters like grain number and weight per plant and starch content. Differential proteomics during flowering stage showed major influence of SA in increasing sink potential with stress acclimation. Stress acclimation was maintained by increased photosynthetic rate and redox homeostasis with increased assimilate allocation and N remobilization to developing sink for yield stability. SA and redox signaling interplay occurred through thioredoxins which activated defense responses. Plants on rehydration strove to maintain the growth at par with well watered plants by constitutive increased expression of proteins involved in photosynthesis, metabolism and defense. To the best of our knowledge, this is the first study on the proteomic mechanism of the SA-induced drought stress tolerance in wheat studied at vegetative and reproductive growth stages. This could be used in future studies focusing on the ameliorating effects of SA in wheat from drought with major goal towards increasing yield potential under stress.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.05.011.

Conflict of interest

Authors declare that there is no conflict of interest.

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