1   **Physiological, Proteomic, and Biochemical analysis Reveal Possible Cross-Tolerance in**

2   **metabolism and heat response proteins in response to Heat and Water Stress in Soybean**

3   Ramesh Katam[1]*, Nitya Murthy[,1,2], Sedigheh Shokri[,1,3], Suresh Lokhande[4], Prashanth

4   Suravajhala[5,6] Mudassar Nawaz Khan[7], Mahya Bahamani[8,9], Katsumi Sakata[10] Vanamrung

5   Isaragumpot[1], and Kambham Raja Reddy[4]*

6   [1]Department of Biological Sciences, Florida A&M University, Tallahassee FL 32307 USA;

7   [2]Kentucky College of Optometry, University of Pikeville, Pikeville, KY 41501 USA; [3]Department

8   of Horticulture Sciences, College of Agriculture, Tarbiat Modares University, Tehran, Iran;

9   [4]Mississippi State University, Mississippi State MS 39629 USA; [5]Bioclues.org; [6]Department of

10   Biotechnology and Bioinformatics, Birla Institute of Scientific Research, Jaipur 302001, India;

11   [7]Institute of Biotechnology & Genetic Engineering, University of Agriculture Peshawar, Pakistan;

12   [8]Department of Agronomy and Plant Breeding, College of Agricultural Sciences & Engineering,

13   University of Tehran, Tehran, Iran; [9] Current address: Department of Biology, School of Science,

14   Edith Cowan University, Joondalup, Perth Australia; [10]Department of Life Science and

15   Informatics, Maebashi Institute of Technology, 460-1 Kamisadori, Maebashi, Gunma 371-0816,

16   Japan.

17   *****Corresponding Authors

18   *****Ramesh Katam Ph. D., Telephone: 850-561-2740; Fax: 850-561-2740

19   Email: Ramesh.katam@famu.edu

20   *****Kambham Raja Reddy, Ph. D. Telephone: 662-325-9463; Fax: 662-325-9461

21   Email: krreddy@pss.msstate.edu

22   **Running Title:** Soybean leaf proteome responses to multiple abiotic stresses

Abstract:

Water stress (WS) and heat stress (HS) have a negative effect on soybean plant growth and crop productivity. During WS, soybean plants opt for survival through ion homeostasis and the conformations of proteins are disconcerted as plant cells lose water while HS leads to difficulties in flowering and fruiting. Some of these changes include oxidative stress leading to the destruction of photosynthetic apparatus, macromolecules within cells and the onset of complex signaling cascades. Changes in the physiological characteristics, proteome, and certain metabolites investigated on molecular and cellular functions were studied in two soybean cultivars exposed to different heat and water stress conditions independently and in combination. Leaf protein composition was studied using 2-DE and complemented with MALDI TOF mass spectrometry. While two cultivars displayed genetic variation in response to water and heat stress, thirty-nine proteins were significantly altered in their relative abundance in response to WS, HS and combined WS+HS in both cultivars; a majority of them involved in metabolism, response to heat and photosynthesis showing significant cross-tolerance mechanisms. Functional analysis revealing a majority of heat responsive-proteins were more abundant during HS and combined stress (WS+HS) whereas these proteins were low to WS in cultivar PI 471938 and heat shock proteins were in low abundance to water, heat and combined stresses in cultivar R95-1705. Most protein abundances were not correlated with their expression at mRNA levels in PI cultivar, however, in cultivar R 95, the expression levels of transcript follow their relative abundance in proteins. Our systems bioinformatics analyses revealed that MED37C, a probable mediator of RNA polymerase transcription II protein showed potential interacting partners in *Arabidopsis* and our studies signifies the marked impact of this protein in PI cultivar. Elevated activities in antioxidant enzymes indicate that the PI-371938 cultivar has the ability to restore the oxidation levels and sustain the

47   plant during the stress. Our study hypothesizes the plant's development of cross-stress tolerance

48   which will help foster the ongoing ventures in genetic modifications in stress tolerance.

49   **Keywords**: antioxidant activity, cross-tolerance, *Glycine max*, heat stress, proteomics, water

50   stress.

51   **<u>Introduction:</u>**

52   *Glycine max* (L.) Merr (soybean) is a legume that provides a significant source of proteins

53   and fatty acids in both human and animal diets. It is an important legume crop grown for its

54   combustion fuel, cooking oil, and protein with over 121.5 million hectares worldwide (1). It is the

55   largest source of feed protein in the world and the second-largest source of food oil (2,3). Soybean

56   is widely adapted and cultivated crop across the climatic zones of the world. The crop plays a

57   significant role in contributing to soil fertility as they are naturally capable of fixing atmospheric

58   nitrogen and the root exudates of some legumes can solubilize phosphorus and other insoluble

59   calcium-bound phosphorus compounds (4,5). The presence of legumes ameliorate the soil quality

60   by encouraging microbial activity, especially around its rhizosphere, by contributing to organic

61   matter restoration, and play a role in disease prevention and pest control (6).

62   Soybean has also been an important model crop for C_3 annual plants, because of its strong

63   response to climate change. For example, a 17% decrease in yield for 1°C rise in temperature have

64   been observed (7). The overall production of soybean is severely limited by several abiotic factors

65   that include flooding, drought, salinity, and acidity (8). Due to its various developmental stages,

66   the abiotic factors strongly impact the plant's growth. Therefore, it is essential to protect crop

67   yields from higher and more frequent episodes of extremely high temperatures and drought both

68   in current and future climates. Water or heat stress is involved in cell dehydration and affects

69   various metabolic functions in plants. Water stress (WS) is one of the most debilitating factors of

70 soybean crop with dehydration in plants leading to a disruption in the water potential gradients,

71 loss of turgor pressure, denaturation of proteins, leading to a lack of investigation in understanding

72 the cellular membranes (9,10).

73   Another devastating effect of dehydration is desiccation, in which the protoplasmic "free

74 water" is lost, and the cell is required to survive on the water-bound within the cell matrix (11). It

75 has also been found that plants respond to dehydration by alternating levels of protein synthesis

76 and protein degradation, with a recent evidence suggesting that there is a direct correlation between

77 the accumulation of proteins synthesized by dehydration stress and the plant's physiological

78 adaptations to water stress (12,13). Besides, when soybeans have encountered water stress during

79 the reproductive stage, owing to a lack of plasticity to recover at this stage, there showed much

80 detrimental decrease in seed yield and its attributes, compared to the plants grown under irrigated

81 condition (14).

82   Heat stress (HS) in the form of high temperatures during flowering, is a cardinal factor

83 limiting seed count in many crops, including soybean (15-18). High temperatures are found in

84 many southern regions of the United States during the germination season of soybean plants.

85 Temperature above 30°C affect germination by decreasing the seed vigor of the soybean. As a

86 result, the levels of stachyose and phytic acid in soybean seeds are decreased, which leads to

87 difficulties in membrane biogenesis and germination (19). While it has been reported that exposure

88 to elevated temperatures encourages oil and protein production in the soybean plants, extreme

89 temperatures result in changes to the seed oil concentration particularly in the ratios of singular

90 fatty acids to total fatty acids in the soybean oil (20). High temperatures can also lead to desiccation

91 of the seeds and cause abnormal exine structure during microsporogenesis resulting in pollen

92 malformation (21). Heat stress tolerance is controlled by adjustments in the membrane structure

93   and function, tissue water content, protein composition, lipid activity, and by primary and

94   secondary metabolites (22). Several reports showed changes in molecules in response to water and

95   temperature stresses at transcription and protein levels that affect photosynthetic efficiency, and

96   the activity of nitrate reductase. The levels of soluble proteins in soybean cultivars were directly

97   correlated with the leaf rate of photosynthesis (23).

98   In response to drought, proteins involved in photosynthesis, signaling pathways, and

99   reactive oxygen species detoxification were severely impacted (24). Both heat and water stress in

100   soybean induced overexpression of *DREB1* family genes, several dehydrins, and LEA genes

101   resulting in their over-representation among up-regulated genes in soybean plants under heat and

102   drought stresses (25). In soybean, most of the differentially abundant proteins are related to

103   photosynthesis, ATP synthesis, and protein biosynthesis (26) even as gene expression of different

104   proteins is directly influenced by WS treatment. For instance, the cellular and biochemical

105   components triggered by drought results in the activation or suppression of specific genes, and

106   consequently the proteins involved in cell division, cell growth, and cell differentiation are

107   affected(27). In response to heat stress, overexpression of enzymes involved in homeostasis, as

108   well as the accumulation of various chaperone proteins, especially heat-shock proteins, were

109   observed (28). The rhizomes of soybean plants have shown that dehydration has a negative effect

110   on the levels of proteins that are responsible for protein transport and storage, ATP synthesis,

111   metabolism, and signal transduction (29). Therefore, proteomic analysis under multiple stresses

112   will lead to better determination of the molecular pathways and the molecules associated with the

113   complex cross-tolerance.

114   Earlier studies described relative abundance in leaf protein composition under water or heat

115   stress conditions in various crop plants (30; 26). However, the interactive effects and complex

116   cross-tolerance mechanisms associated with physiological, biochemical, and proteome changes to

117   heat and water stress are not well understood. As plants undergo a combination of multiple stresses

118   in the field condition, they trigger defense mechanisms and cooperative systems, which under

119   multiple stresses display cross-tolerance with each other to increase the plants' immune efficiency

120   (31). Cross-tolerance is a phenomenon in plants that makes them become more tolerant to second

121   stress after imposing under first stress, such as induction of stress memory after the stress (32).

122   Simultaneous occurrence of more than one stress can have both positive and negative impacts on

123   the plants performance and adaptation (33). Therefore, determining the key regulators that take

124   part in orchestrated responses to concurrent stresses provide better understanding of tolerance

125   mechanisms. The objective of present study was to assess the effect of water stress, heat stress,

126   and combined stresses on the regulation of leaf proteins in two contrast soybean cultivars.

127   **Materials and methods:**

128   *2.1: Experimental facility*

129   The study was conducted in four sunlit, Soil-Plant-Atmosphere-Research (SPAR) plant growth

130   chambers located at Mississippi Agriculture and Forestry Experiment Station, Mississippi State,

131   MS, USA (www.spar.msstate.edu). Chamber air temperature, CO_2, and soil watering were

132   controlled to provide automatic acquisition and storage of the data from the units, monitoring

133   SPAR environments every 10 seconds (34,35). The high temperature, 38/30°, and drought stress

134   at optimum and high temperatures were imposed by withholding irrigation until the soil water

135   content reached 96% (-3.0 M Pa) of the control (-1.5 M Pa). Leaf samples were collected from the

136   plants for proteome analysis.

137   *2.2: Plant growth and treatments*

138   Two contrasting soybean cultivars - PI 471938 (slow wilting and high yielding; Cultivar#2)

139   and R95 – 1705 (high in protein concentration and moderate yield potential- Cultivar#1) were used

140   in the experiment. The detailed experimental procedure outline is shown in Figure 1. Soybean

141   seeds for both cultivars were planted in three rows. Plants were thinned to 10 plants per row 12

142   days after emergence (DAE) and irrigated three times a day with half-strength Hoagland's nutrient

143   solution (36). The air temperature of 28/20°C (day/night) was maintained until the beginning of

144   the treatments (30 DAE). After that, four treatments consisting of two levels of each factor,

145   temperature (28/20° and 38/30°C) and irrigation (well-watered-WW, and water stress-WS) were

146   imposed until the harvests (57 DAE). The control treatments consisted of 28/20°C and WW. The

147   WS was imposed gradually as follows: no irrigation (30-31 DAE), watered 40% of the control (32-

148   39 DAE), and no irrigation (40-50 DAE). The high temperature, (38/30°) and drought stress at

149   optimum and high temperatures were imposed by withholding irrigation until the soil water content

150   reached 96% (-30 M Pa) of the control (-1.5 M Pa).

151

152   Figure 1: Experimental outline, deciphering the analysis of physiological and biochemical changes
153   in response to stress in soybean
154   *2.3: Physiological measurements*

155   *a. Soil water content:* The soil water content (SWC) was measured with a soil moisture

156   probe (Delta-T Devices, Burwell, UK). Leaves of soybean plants were detached to measure the

157   leaf's fresh, turgid and dry weights, and the relative leaf water content was determined as follows:

158   LWC = (fresh weight – dry weight)/ (turgid weight – dry weight).

159   *b. Chlorophyll content:* Total chlorophyll was extracted by placing five 0.38 cm^{-2} leaf disks

160   for each row in a vial containing dimethyl sulfoxide (5ml) and incubated in the dark for 24h.

161   Afterthat, the absorbance of the supernatant was measured using a UV/VIS spectrophotometer

162   (Bio-Rad Laboratories, Hercules, CA, USA). The total chlorophyll was estimated and expressed

163   on leaf area basis (37).

164   *c. Measurement of gas exchange and chlorophyll fluorescence:* Gas exchange and

165   chlorophyll fluorescence parameters were measured simultaneously using a Li-Cor 6400

166   Photosynthesis system (Li-Cor Inc., Lincoln, Nebraska, USA). The values of P_{net}, Fv′/Fm′,

167   stomatal conductance (g_s), intercellular (C_i) CO_2 concentration, and transpiration were

168   automatically computed from the instruments software.

169   *2.4. Statistical analysis*

170   Statistical analyses were performed using SAS (SAS Enterprise Guide, 4.2, SAS Institute

171   Inc., NC, USA). PROC MIXED with Kenward-Rogers (kr) adjustment of degrees of freedom was

172   used for analysis of variance (ANOVA) to test the effect of treatments and cultivars, and their

173   interactions on the plant and soil water status, chlorophyll concentrations, gas exchange and

174   fluorescence parameters, and total biomass. Treatments (temperature and irrigation) and cultivars

175   were considered as the fixed effect, and individual measurements / rows were the random effects.

176   The treatment comparisons were conducted by a least-square means (LSMEANS) procedure (at

177   α=0.05) and the letter grouping was obtained using pdmix800 macro (38).

178   *2.5. Total protein extraction of soybean leaf*:

179   The uppermost fully expanded leaves were detached and collected into liquid nitrogen. Leaves

180   were collected from six plants of the same cultivar then frozen in liquid nitrogen and stored at -

181   80° C before protein extraction. Protein was extracted following the modified procedure (39).

182   Briefly, frozen powder (6 g) was vortexed in 20 ml of 50 mM Tris HCl (pH 7.5) containing 2 M

183   thiourea, 7 M urea, 2% Triton X-100, 1% DTT and 4% PVPP. The suspension was centrifuged at

184   5000 rpm and the protein was precipitated with TCA (15%). The protein pellets were washed twice

185   in cold acetone (-20° C) and centrifuged for 15 min at 13000 rpm. Final pellets were resuspended

186   in IEF rehydration solution [7 M urea, 2% CHAPS (w/v), 2 M thiourea, 0.2% DTT (w/v)] to

187   measure the protein concentration (40).

188   *2.6. 2-DE Protein Mapping:*

189   An aliquot (300 µg in 100 µl) of the protein extract was loaded on to the tube gels and

190   isoelectric focusing (IEF) was performed as described previously (41). Tube gels were then loaded

191   on a slab gel, and the proteins were resolved by electrophoresis.

192   *2.7. Gel image and Statistical analysis:*

193   Gels were scanned using a Gel Image system (Bio-Rad, Hercules, CA). The Analysis Set

194   derived from three replicated gels of matched spots that were present on all the gels and the three

195   replicated gels were analyzed using PD Quest (version 8.0.1). A one-way analysis of variance

196   (ANOVA) was conducted to compare the mean protein spot densities and test if there was any

197   difference in the protein spot abundance among the treatments and two cultivars studies. The

198   differentially expressed spots (with P-values <0.05) showing significant differences were chosen

199   for further analysis. Protein spots were manually excised from gels following in-gel digestion and

200   MALDI/TOF mass spectrometry.

201   *2.8. In-Gel Trypsin Digestion:*

202   The in-gel digestion mixture was a disulfide bond reduction. The resulting peptide mix was

203   desalted with C_{18} Zip Tips (Millipore), and 0.7 µl of the eluate and 5mg/ml matrix (α-cyano-4-

204   hydroxycinnamic acid) was spotted on the ABI 01-192-6-AB MALDI plate (Applied Biosystems,

205   Foster City, CA).

206   *2.9. Mass Spectrometry, Database Search, and Protein Identification:*

207   Mass spectra were collected on the ABI 4700 Proteomics Analyzer (Applied Biosystems)

208   MALDI/TOF mass spectrometer (MS), and protein identification was performed using the

209   automated result dependent analysis of ABI GPS Explorer software, version 3.5 (Applied

210   Biosystems). Data were analyzed as Peptide Mass Fingerprinting (PMF), and protein

211   identifications weredone by searching against the database using the MOWSE algorithm (42).

212   Both MS and MS/MS data were matched against Phytozyme the soybean taxonomic database.

213   Only the proteins with a total score of confidence interval (C. I) % > 95% were considered as

214   positive identities.

215   *2.10. Gene Ontology (GO) Annotation:*

216   The identified proteins were mapped to Universal Protein Resource (UniProt KB) to assess their

217   functional analysis as previously described (43). The accessions were queried using batch Entrez

218   to retrieve several sequences that mapped to different proteins. The annotations and accession

219   numbers were retrieved using the GO Retriever tool and were grouped into different levels. Protein

220   sequences were searched against gene ontology tools and the Target P program to derive functional

221   classification, cellular localization and further validated using MapMan bin codes (44).

222   *2.11. Putative protein-protein interaction networks of differentially expressed proteins to WS+HS*

223   *stresses:*

224   Time course expression data was used to estimate the interaction among the proteins in

225   both cultivars. Protein-protein interactions (PPI) were estimated by temporal expression profiling

226   utilizing an *S-system* differential equation [R1] as previously described (45). Furthermore, from

227   the association studies, 39 proteins were interolog mapped to *Arabidopsis* database using

228   GeneMania (46).

229   *2.12. Quantitative Real-Time Polymerase Chain Reaction (RT-q PCR) Analysis:*

230   Total RNA was isolated using a modified CTAB-based protocol for RT-qPCR (47). A

231   NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and agarose

232   gel electrophoresis were used to test RNA quality and quantity. Total RNA from each sample was

233   reverse-transcribed using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Gene-

234   specific primers (Supplementary Table I) were designed with Primer Premier 5.0 (48).

235   *2.13. Enzyme assay:*

236   Superoxide dismutase, Catalase and peroxidase activities were measured as described in the

237   literature (49-51). The leaf tissue was homogenized in 50 mM of KPO_4 buffer (pH 7.0) for

238   ascorbate peroxidase (APX). The supernatant was used for APX assay from the decrease in

239   absorbance at 290 nm (52). The absorbance of non-enzymatic oxidation of ascorbate by H_2O_2 was

240   used as control. The reaction was measured by the decrease in absorbance at 340 nm. Glutathione

241   reductase (GR) was determined using the protocol (53) by measuring the reduction of GSSG by

242   NADPH and measuring the absorbance at 340 nm.

243   **<u>Results:</u>**

244   Two soybean cultivars were used in this study to determine the changes in physiological,

245   molecular, metabolite and enzyme activities in leaf tissues subjected to water stress or heat stress

246   and combination of both.

247 **1. Physiological measurements:**

248   *Soil water content (SWC), chlorophyll content, measurement of gas exchange and*

249 *chlorophyll fluorescence:* Water stress caused a severe decrease in soil water content (SWC) and

250 relative leaf water content (LWC) leading to a reduction in photosynthetic rate (P_{net}), stomatal

251 conductance (g_s) transpiration (Tr) and total biomass (TBM) in both cultivars. In plants grown at

252 28/20°C under well water (WW) conditions, the plant responses to water stress (WS) was more

253 severe than high temperature (38/30°C) with the exception of the stomatal conductance (g_s) and

254 the transpiration (Tr) which increased 33-39 and 71-91% at high temperature, respectively

255 (Table1). Among the physiological parameters, the C   T   IRR and the C   T and interactions

256 were significant ($P \leq 0.01$) for SWC and C_i. The T   IRR interaction was significant ($P \leq 0.01$)

257 for g_s, Fv'/Fm', C_i and Tr whereas, C   IRR interaction was significant ($P \leq 0.05$) for P_{net}, C_i and

258 Tr. The treatments (T and IRR) significantly ($P \leq 0.001$) affected all the parameters except a few

259 incidents of chlorophyll concentration, P_{net}, and TBM. Chlorophyll content was increased in

260 response to combined water and temperature stress in the cultivar PI 471938 while it was reduced

261 in the R-95 cultivar. With the exception of chlorophyll concentration in both cultivars, water stress

262 in combination with high temperatures showed a greater reduction in these parameters. Water

263 stress caused 14% reduction in leaf water content in both cultivars, while it was further reduced

264 by 21% when both stresses were imposed. Compared to the control (well-watered), water stress

265 severely reduced P_{net} (>86%), g_s (>95%), and transpiration (>89%), either alone or in combination

266 with high temperature in both cultivars. However, high temperature alone increased both g_s

267 (>33%) and transpiration (>70%) in both cultivars. R95–1705 showed greater decrease in TBM

268 (50%) than 471938 (38%) under water stress at high-temperature conditions.

269 **2. Analysis of Stress Responsive proteins:**

270   *i) 2-D Gel Electrophoresis and identification of relative abundant stress-responsive*

271   *proteins*: The 2-DE analytical gels of leaf proteins revealed that most of the proteins had a

272   molecular weight (M_r) between 10 and 66 kDa, and pI between 4.3 and 7.9, a pattern typically

273   observed in most of the leaf tissues (Figure 2). PD Quest digital image analysis and visual spot-

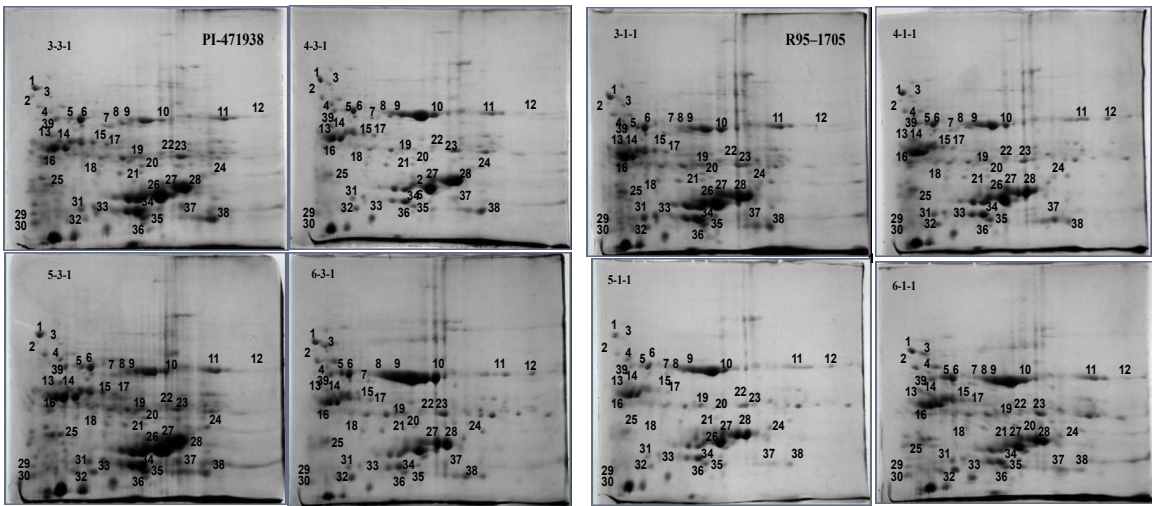
274   by-spot validation of the match derived from 2-DE gels when carried out at a sensitivity reading

275   of 5.0 revealed over 200 proteins in both the cultivars (Supplementary Table II). Cultivar PI

276   373819 showed 25% decrease to WS, an 8% increase to HS and a 10% decrease to both WS+HS

277   in total proteins; while cultivar R-95 showed a decline by 6.6% to WS, 33% to HS, and a 10% to

278   both WS+HS.

279

280   **Figure 2:** Changes in leaf protein abundance (protein spots with numbers) to water (WS), heat
281   (HS) and combined stresses (WS+HS) in soybean cultivars
282

283   Comparative analysis of protein quantification profiles of both cultivars was carried out to

284   identify the relative abundance of proteins in both cultivars to both stress conditions. Its considered

285   >1.5-fold increase or <1.5 fold decrease as threshold level to determine the protein abundance

286   based on the correlation coefficient (r N 0.95), following the procedure published by Su et al 2013.

287   Comparative analysis of protein profiles revealed 39 protein spots, showing quantitative variation

288   following water stress or heat stress that satisfies the 95% confidence interval (Table 2).

289   *ii) Identification and functional classification of relative abundant Stress responsive*

290   *proteins:* All of these 39 protein spots accounted for 31 non-redundant proteins, with eight protein

291   spots (4 proteins) showing similar protein accessions detected in multiple locations with

292   differences in their isoelectric points and/or molecular weights on 2-DE gel. Relative protein

293   abundance to various treatments between two cultivars and close up view of selected protein spots

294   were is shown as in Supplementary Figure I and II. To determine the functional categories related

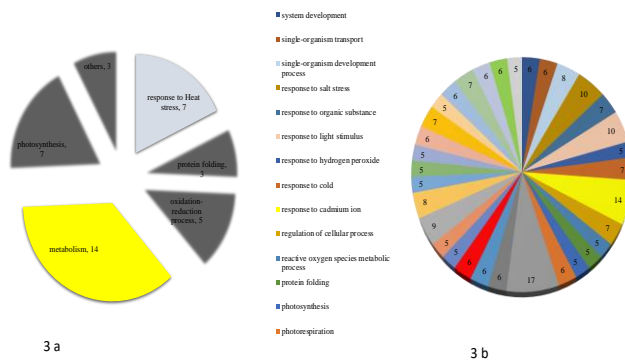
295   to stress responses, the proteins were classified into five major functional categories including

296   metabolism (14), response to heat (7), photosynthesis (7), redox process (5), protein re-folding (3)

297   and others (3) (Figure 3a). Over half of the proteins belonged to metabolism, response to heat and

298   photosynthesis. The molecular function of each protein is shown in Figure 3b.

3 a   3 b

299

300   **Figure 3:** Gene ontology of stress responsive proteins: (A) Biological, (B) Molecular functions
301   Some of the biological functions of the proteins in control and treatments of both cultivars

302   were validated using MapMan (Figure 4, Supplementary Figure III). The protein data were also

303   analyzed to determine their association with individual organelles. The results predicted that they

304   are localized in chloroplast, mitochondria, and others (Figure 5).

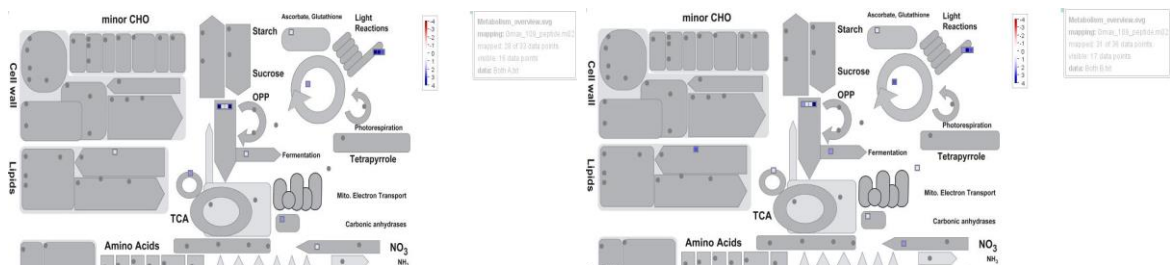
305

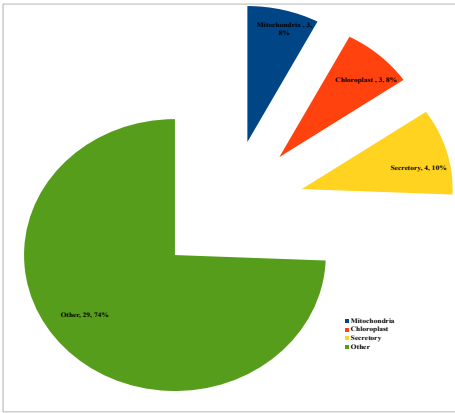
306

307

308

309

310

311   **Figure 4:** Phytozyme confirmations of protein identities using MapMan

312

313   **Figure: 5**: Sub-cellular location prediction using TargetP

314   *iii). Clustering and dynamics of stress-responsive proteins:* Expression analysis of the

315   thirty-nine relatively abundant proteins were carried and analyzed (Figure 6). Four clusters of

316   protein expression were recognized in both cultivars.

317   In cultivar PI 471938 (slow wilting and high yielding) I clustered proteins that were

318   increased in abundance to either water stress (WS) or heat stress (HS) or the combination of water

319   and heat stress (WS+HS) (Figure 6a). Out of eight proteins (# 3, 4, 9, 10, 12, 17, 24, and 27) the

320   majority of them are involved in metabolism (3) photosynthesis (2), and response to heat (2) and

321   other (1). Among the proteins in Cluster I, four proteins showed a high abundance to more than

322   two stresses (#4 10, 17 and 27) of which, two proteins (#10 and 17) showed increase in abundance

323   to all three types of stresses. Cluster II includes 19 proteins (#2, 5, 6, 7, 13, 14, 15, 19, 20, 21, 22,

324   23, 26, 29, 30, 34, 37, 38, 39) that are in low abundance in response to at least one or more of the

325   three stress types. The majority of these proteins involved in metabolism (9), oxidation-reduction

326   reactions (5). Fifteen proteins in cluster II were low in abundance to two or more stresses. Seven

327   of them (#7, 13, 15, 21, 22, 29, 38) were low in abundance to all three types of stresses; the majority

328   of them were involved in metabolism. None of the proteins fall under the category of Cluster III

329   in PI cultivar. Cluster IV includes 12 proteins (#1, 8, 11, 16, 18, 25, 28, 31, 32, 33, 35, 36)

330   exhibiting mixed responses to each stress type/s, with the majority of them were involved in

331   response to heat (3), and photosynthesis (3).

332   In cultivar R-95 (high in protein concentration and moderate yield potential), cluster I

333   displayed a group of 10 proteins increased in their abundance to at least one type of stress, either

334   to WS, or to HS, or the combined WS+HS (Figure 6b). Those proteins (#3, 4, 7, 8, 9, 12, 14, 23,

335   24, and 25) are mainly involved in metabolism (5), following photosynthesis (3), response to heat

336   (1), and oxidation-reduction reactions (1). Among these proteins five of them showed resistance

337   to two or more stresses involved in metabolism and photosynthesis. Four proteins showed high

338   abundance to WS, and three to HS. Protein # 7 and 12 involved in metabolism were not present in

339   control but expressed in different quantities to all stresses studied. Cluster II includes 22 proteins

340   that are in low abundance to one or more of the stresses (either WS, or HS or WS+HS) treatment

341   (Spot #1, 2, 6, 10, 11, 16, 17, 18, 19, 22, 26, 27, 28, 29, 30, 32, 33, 34, 35, 36, 38, 39) and include

342   those involved in response to heat (5), photosynthesis (4), metabolism (4), protein refolding (3),

343   oxidation-reduction (3), and others (3). Among these, sixteen proteins showed reduction in

344   abundance to two or more stresses, majorly involved in response to heat. ten proteins showed

345   reduction in abundance to all three stresses. Cluster III proteins (spot # 20, and 21), which are

346   involved in metabolism showed no response to water stress, heat stress, and combined WS+HS

347   stresses. Cluster IV includes six proteins (Spot #5, 13, 15, 22, 31, and 37), mostly involved in

348   metabolism (4) which showed mixed response to the stresses, low abundance to one type and high

349   to another type stress.

PI-471938   R95-1705

350

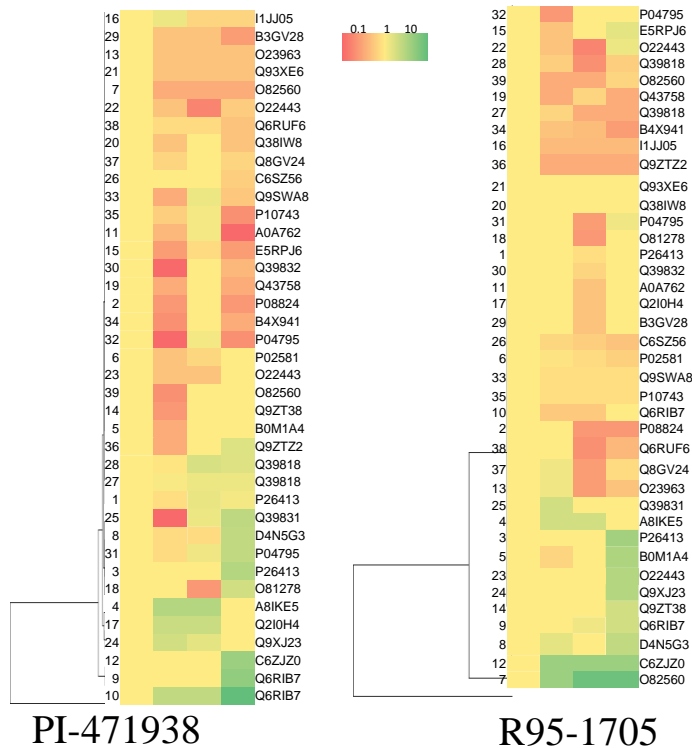



Figure 6: Relative abundance profile of 39 stress responsive proteins (SRPs) in a) PI and b) R-95
cultivars as depicted in hierarchical cluster.
The protein abundance ratios were used for cluster analysis by a hierarchical clustering method (centroid
linkage Protein abundance ratios were used for cluster analysis by a hierarchical clustering method (centroid
linkage based on Euclidean distance metric). Columns (from left to right): Control, Water stress, Heat stress,
and Combination of water and heat stress.

iv). Putative PPI networks of differentially expressed proteins to abiotic stresses: Relative

abundance of stress responsive-proteins were studied in time-course expression for the combined

treatment of water and heat stress to determine the interactions among the proteins in both

cultivars. Time course expression data was used to estimate the interaction among the proteins in

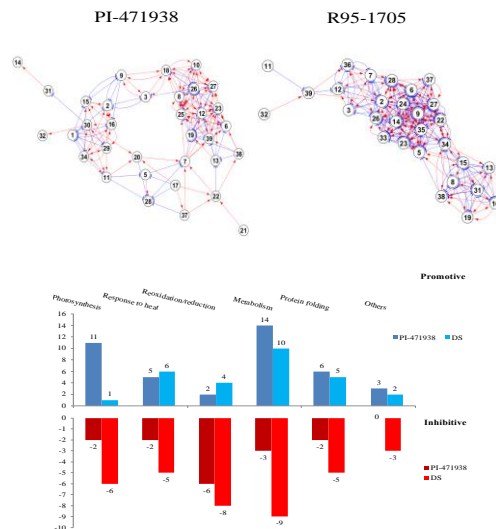
both cultivars to understand the interaction levels of proteins to the biological function in response

363   to both WS+HS stress over a 3-week duration. Thirty-two proteins have shown interaction in the

364   PI cultivar, while 29 have interacted in R95 cultivar. In PI cultivar, 95 promotive and 61 inhibitive

365   interactions were observed, while 113 promotive and 139 inhibitive interactions were observed in

366   R95 cultivar (Figure 7; Supplementary Table III).

367

368   **Figure 7:** Protein interactions among SRPs.

369

370   A: Protein interactions in PI cultivar showing more interactions compared R95. The number of
371   interacting proteins involved in various biological functions is shown in C. More proteins involved in
372   protein synthesis and metabolism are associated with interactions in PI cultivar than R95. D. The estimated
373   interactions were evaluated based on a goodness-of-fit calculated from "multiple-correlation coefficient"
374   (R^2) between an expression profile and simulated profile of a protein positioned on the downstream side of
375   an interaction. The interactions showing an r2 value (coefficient of determination) >0.9 were considered as
376   candidate interactions. We calculated the R^2 corresponding to the interaction that a protein regulated the
377   expression of another protein based on a modified version of the S-system differential equation. Spot
378   numbers are the same as in Table II.

379

380   To further validate the interaction, the proteins that showed high abundance to combined

381   WS+HS were also analyzed to determine the frequency of promotive and inhibitory interactions

382   in both cultivars. PI 471938 showed high abundance of 11 proteins belonging to heat resistance

383   (3), oxidation-reduction (2), metabolism (5), and photosynthesis (2) to combined WS+HS

384   (Supplementary Table IV). These proteins displayed 41 promotive and 15 inhibitive interactions.

385 Among the promotive interactions, the majority were involved in metabolism, response to heat

386 and photosynthesis. Cultivar R95–1705 showed a high abundance in 8 proteins involved in

387 resistance to heat (4), photosynthesis (2), metabolism (1), and others (1) (Supplementary Table V).

388 These proteins displayed 28 promotive and 36 inhibitive interactions. Among the promotive

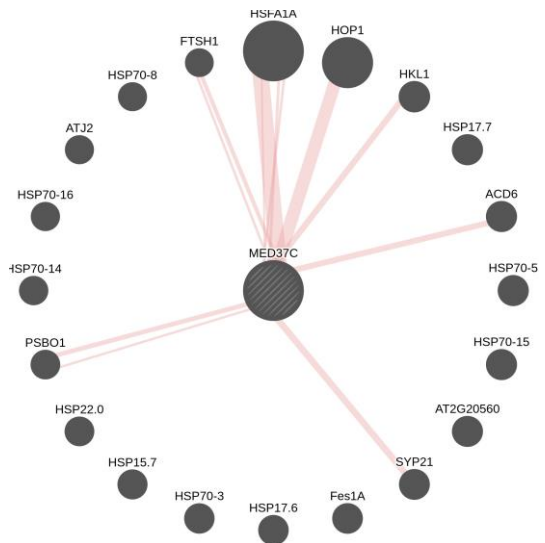
389 interactions, majority were involved in metabolism.

390   When the 39 SRPs were subjected for interolog mapping and protein interaction analysis,

391 heat shock protein 70 (MED37C) showing potential interactions with stress-related proteins

392 indicate that these orthologs in *Arabidopsis thaliana* genome have good potential interacting

393 partners (Figure 8).

394

395 **Figure 8:** Protein-protein interaction map of the gene MED37C

396 Physical interactions shown in the form of edges (Thicker the edge, greater the number of experiments for
397 which interactions were ascertained).

398

399 **3. Comparative studies of mRNA transcript analysis with protein expression:**

400   Selected stress-responsive proteins (SRP) were selected for transcriptional level expression

401 analysis. Leaf samples from control and stressed plants were collected for mRNA extraction and

402 then analyzed for RT qPCR. Results showed that, in cultivar PI 471938, the mRNA levels of

403   ascorbate peroxide, chalcone flavone isomerase, serine hydroxymethyl transferase 5, calreticulin

404   (CA), peroxidase, heat shock protein 70, superoxide dismutase showed up-regulation to all stress

405   treatments, while catalase and peroxiredoxin showed reduced levels to HS treatment (Figure 9a).

406   In R95-1705 cultivar, majority of the transcripts showed mixed responses (Figure 9b). Catalase

407   and serine hydroxymethyl transferase 5 showed up-regulation of transcripts while calreticulin

408   (CA), heat shock protein 70, peroxidase, peroxiredoxin and superoxide dismutase showed down-

409   regulation to all stress treatments. Expression of ascorbate peroxide increased to HS while reduced

410   to WS, and WS+HS treatments. Chalcone flavone isomerase levels were increased to WS and HS

411   while this transcript did not express at combined stress treatment.

412

413

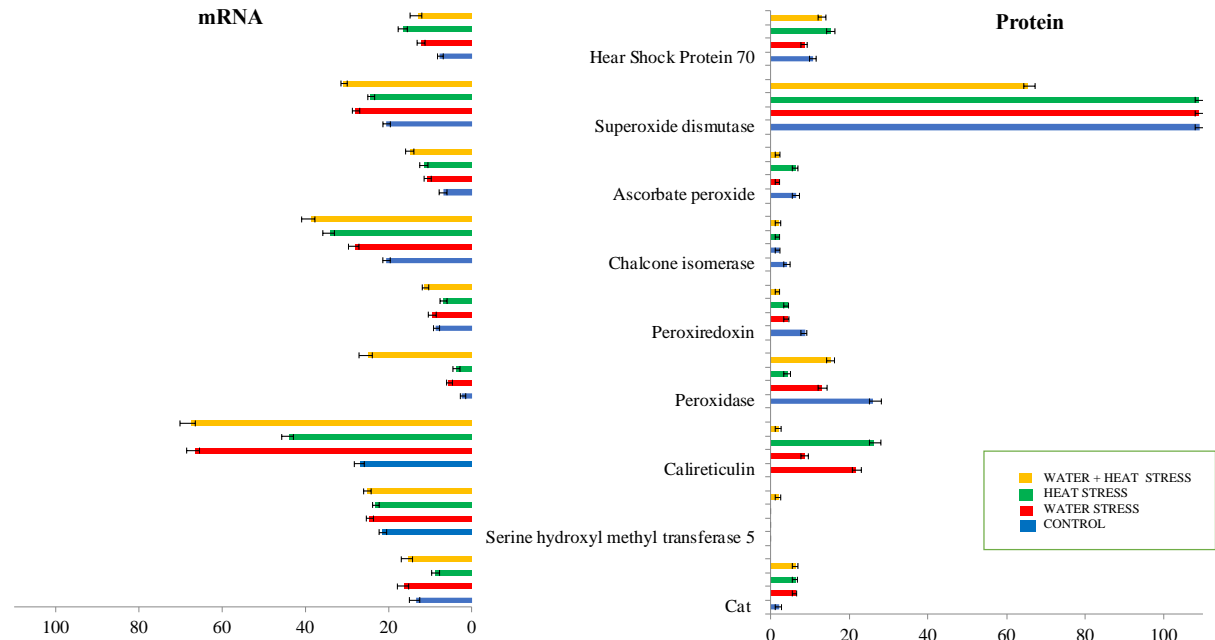
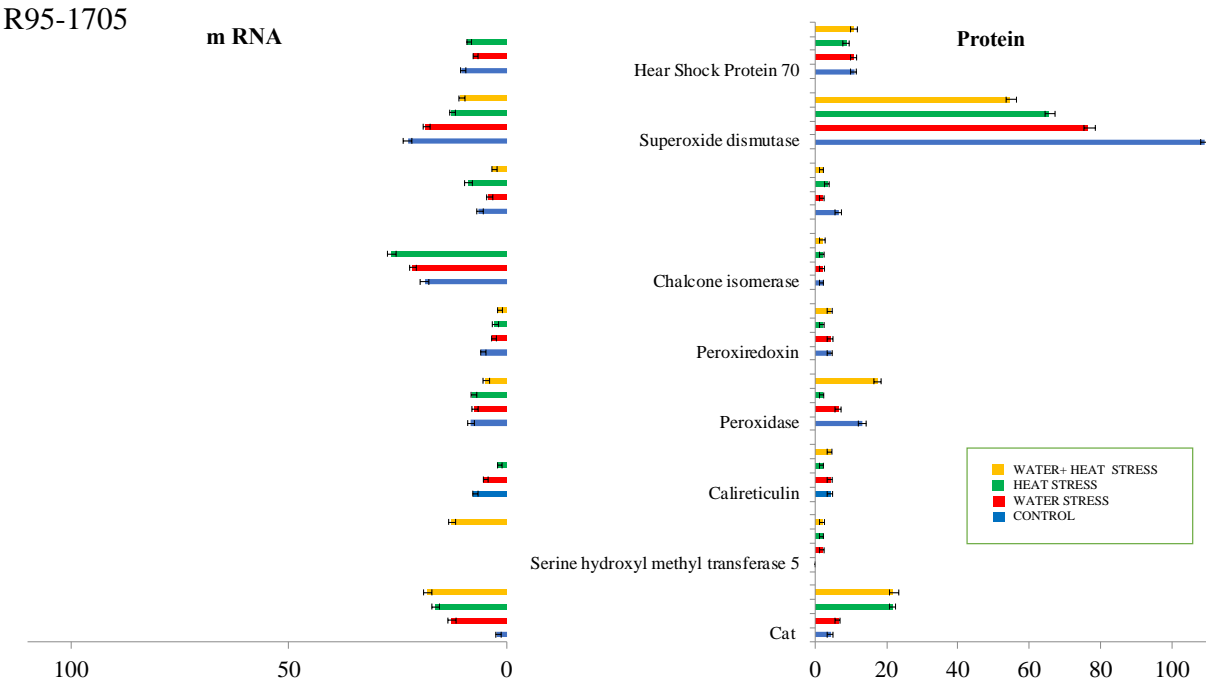



Figure 9: Changes in transcript expression and their protein abundance in response to various stresses in in soybean leaf of (A): PI and (B): R95-1795 soybean cultivars.

Relative mRNA abundances were normalized against actin gene abundance. Stress 1: Water Stress; Stress 2: Heat Stress, and Stress 3: Water + Heat stress respectively. RT-qPCR was performed on Bio-Rad iCycler using the cDNA product corresponding to 20 ng of total RNA in a 20 μl reaction mixture, that includes 1μL of forward and reverse primers of the transcripts, for selected genes using SYBR green method. PCR conditions were: 95° C for 30 s, then 45 cycles of 95° C for 10 s and 60° C for 30s. Data was acquired at 60°C. Data was normalized using the Actin gene Ct value, and extent of change was calculated using the Ct value of the calibrator (control samples -no stress treatment) using the formula 2-ΔΔCt.

4. Determination of enzymes activities:

The enzyme activities in both cultivars were measured for both control and stress treated plants in replicates. Under control conditions, relatively, the activity of superoxide dismutase (SOD; EC 1.14.1.1), peroxidase (POD EC.1.11.1.7), and catalase (CAT, EC 1.11.1.6), were low in PI 471938 cultivar compared to those in R95-1705, whereas ascorbate peroxide (APX), glutathione reductase (GR) showed higher activity in PI cultivar (Figure 10). The activity of SOD, POD, APX and CAT was significantly increased in PI while it was reduced in R95 cultivar when treated with WS, HS, and WS+HS. The activities of GR were significantly higher in all three types of stresses in both

433   cultivars, with an exception that GR has not been detected in R 95 cultivar when treated with

434   combined stresses.

435

436

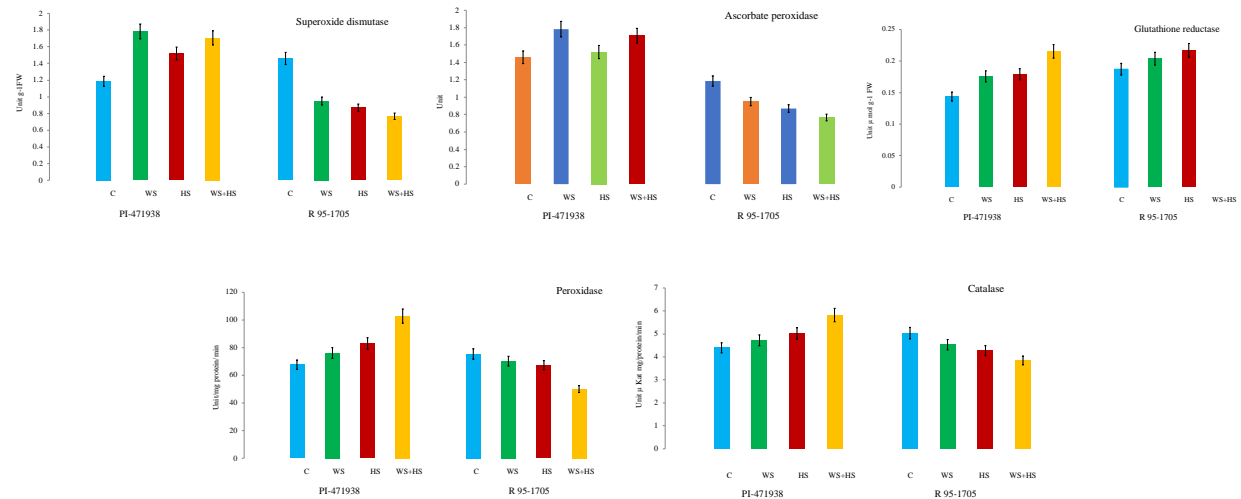



Figure 10: Enzymatic activities under water stress, heat stress and the combined stress.
(The activity is measured in terms of fold change over the control; C: Control; WS: Water stress; HS: Heat stress; WS+HS: Water and heat stress).

Discussion:

442   Plants' responses to concurrent stresses often occurring in the field are very exclusive,

443   when compared to individual stress treatments and will display cross-tolerance to better adapt to

444   those stresses (54-55). We report the result of our studies on two contrast soybean cultivars to

445   determine the physiological and molecular mechanism for cross-tolerance between multiple

446   stresses.

447   *i) Greater reduction in Total biomass to stress treatments in R95-1705 cultivar:* The

448   observed decrease in photosynthetic rate (P_{net}), stomatal conductance (g_s), transpiration (Tr) and

449   total biomass (TBM) under water stress alone or in combination with high temperatures was

450   closely associated with decrease in soil and plant water status. In both cultivars, the impact of

451   water stress was several folds greater than the effect of high temperature, when these stresses are

452   applied independently. WS, HS, WS+HS reduced stomatal conductance which is caused by leaf

453   water potential via transpiration rate alterations in both cultivars. Photosynthesis is among the

454   primary processes affected by WS+HS (56). No significant reduction in photosynthesis was

455   observed during HS alone, whereas the consequences of water deficiency due to WS or WS+HS

456   have more significant impact on altering photosynthetic machinery in both cultivars. Both stomatal

457   conductance (g_s) and transpiration (Tr) were severely reduced under water stress, accompanied by

458   reduction in the soil and plant water status. Plants partially close stomata to reduce the net

459   transpiration under water stress, which might lead to the observed severe decrease in the

460   photosynthesis (P_{net}) (57). However, the combined effect of two or more stress factors including

461   high temperature has been reported to be more deleterious than the effect of a single stress factor

462   on plant growth (58). Earlier studies showed reduction in photosynthesis and dry matter

463   productions due to water stress and high temperature in soybean (59-60; 14). Compared to the

464   control, despite the lesser reduction in P_{net} and leaf water content, cultivar R95–1705 showed

465   greater decrease in the total biomass than in PI 471938.

466   Changes in proteins and anti-oxidative enzymes implicate the morphological and

467   physiological adaptation in plants to stress (61-62). Hence, the proteomic studies were carried out

468   to evaluate the relative abundance of proteins and correlate with similar enzyme activities using

469   two contrasting soybean cultivars in response to drought stress. Furthermore, we studied the effect

470   of individual and combined stresses to water and heat stress and to determine the molecular

471   mechanism for cross-tolerance between multiple stresses. Genetic variation was observed for

472   responses to two stresses. PI cultivar was affected to WS displaying a 25% reduction in proteins

473   compared to R95 cultivar, whereas, R95 exhibited a greater reduction in response to HS in proteins.

474   Reduction in proteins was reported in wheat heat-sensitive cultivar compared to tolerant wheat

475   (63). However, when combination of WS+HS was administered, a similar reduction rate in the

476   number of proteins was observed in both cultivars. Identification of protein isoforms with the same

477   accession numbers spotted at multiple locations may be present due to alternative splicing,

478   polymorphism, and post-translation modifications and they add to the proteome complexity (64).

479   Often the protein isoforms differ in their cellular concentration and can be used as biomarkers (65).

480   *ii) Dynamics of stress-responsive proteins indicates PI-471938 cultivar, a heat-tolerant:*

481   Cluster I proteins that showed high abundance to one or more stresses, in contrast to cluster II

482   proteins which showed low abundance to one or more stresses are represented in almost identical

483   numbers in both cultivars, and the majority are metabolism proteins. Among cluster II proteins, a

484   greater reduction in abundance of heat response proteins and photosynthesis related proteins were

485   observed in the R 95-1705 cultivar. In R 95-1705, eleven proteins were reduced in abundance to

486   all three types of stresses, in particular, affecting more heat response proteins followed by redox

487   proteins. In PI cultivar, seven proteins showed reduced abundance in all three stress types,

488   primarily involving in metabolism and redox proteins. Cluster III proteins were all involved in

489   metabolism (spot # 18, 20, and 21) showed no response to either water, or, heat, or combined

490   stresses in R 95, while they were low in abundance in PI cultivar.

491   Cluster IV proteins that displayed mixed responses included twelve proteins from PI

492   cultivar and six proteins from R-95 cultivar. In PI cultivar, ten proteins showed potential cross-

493   tolerance, five with water and the combined stresses, and five with heat stress and the combined

494   stress, the majority of them are heat response proteins. Heat response proteins that were overly

495   abundant to combined stress in PI were also more abundant to HS, while two of them were low

496   abundant to WS, suggesting that the cultivar is more responsive and tolerant to HS and to combined

497   HS+WS stress than WS alone.

498   The stress memory to one stress may prevent damages accruing from other stresses (66).

499   Plants use stress memory to stabilize performance when exposed to infrequent environmental

500   changes and increase resilience. Our studies revealed that, in PI cultivar, the majority of proteins

501   involved in heat response and redox had shown either water or heat stress memory. Whereas, in

502   R95 cultivar, the majority of proteins involved in metabolism and photosynthesis have shown

503   either water or heat stress memory, and redox proteins showed heat stress memory in R95 cultivar.

504   Accumulation of transcription factors or proteins facilitates a fast response to repeated stress

505   exposure (67).

506   *iii). Effect of heat stress showed high abundance of the heat-responsive proteins in PI-*

507   *cultivar:* The majority of heat response proteins-heat shock protein 70, 22 kDa heat shock protein,

508   17.7 kDa class 1 small heat shock protein, and 17.6 kDa class 1 heat shock protein, were

509   consistently high in abundance to heat stress in PI cultivar, but were low in R-95 cultivar. HSP70

510   is one of the most critical proteins in the response to heat stress, and studies show that this protein

511   is directly linked to the thermotolerance of the plant (68). HSPs and small HSP make up molecular

512   chaperones and involved in protein folding, prevention of protein aggregation, translocation of

513   proteins across membranes, targeting proteins towards degradation, and regulation of translation

514   initiation, thus the renaturation of stress-damaged proteins protecting cells against the effects of

515   stress (69). Some HSPs are also involved in transcriptional activation of additional small HSP

516   promoters (70). Plants up-regulate HSPs, in particular HSP-70, are more tolerant of heat stress

517   (71).

518   *iv) Effect of water stress showed a high abundance of proteins involved in metabolism in*

519   R95-1705 *cultivar:* Out of the total 12 proteins involved in the metabolic processes, four proteins

520   were relatively more abundant with 5 proteins maintaining their abundance in R95, only two

521   proteins were more abundant in PI, and two maintained their abundance.

522   Alanine aminotransferase 2 was the only protein that was more abundant in response to

523   WS in both cultivars. The protein is found in peroxisomes and involved in the degradation of

524   amino acids in plant cells (72). The abundance of these enzymes indicates that the amino acid

525   metabolism and the synthesis of other metabolites derived from amino acids are well maintained

526   under drought stress (73). Acid phosphatase was overexpressed to WS in R95 and remained

527   unchanged in PI cultivar. The role of acid phosphatase is essential in maintaining metabolic

528   homeostasis during drought stress (74).

529   Serine hydroxymethyltransferase 5, a glycolytic protein involved in photorespiration was

530   not expressed in the control plant however was induced to combination of WS+HS stresses in R95

531   cultivar. These proteins are directly involved in initiation and elongation of the newly growing

532   peptide chains, indicating severely reduced synthetic protein capacity under drought. Serine

533   hydroxymethyltransferase (SHMT; EC 2.1.2.1) is involved in the photorespiratory pathway of

534   oxygenic photosynthetic organisms (75). SHMT, a pyridoxal phosphate-dependent enzyme, plays

535   a pivotal role in cellular one-carbon pathways by catalyzing the interconversion of L-serine to

536   glycine and tetrahydrofolate to 5,10-methylenetetrahydrofolate for synthesis of nucleic acids, and

537   proteins (76).

538   Translation elongation factor Tu was overexpressed to WS in R95 cultivar. Translation

539   elongation factor Tu is a GTPase that is responsible for delivering amino-acylated tRNAs to the

540   ribosome during translation (77). Nucleoside diphosphate kinase (NDPK) is more abundant in R95

541   cultivar, and low in PI in response to WS. NDPK is found in the matrix and the inner membrane

542   of mitochondria, which regulates the cellular physiology, is known to interact with heat shock

543   proteins (78).

544   *v) Combined water and heat stress significantly altered metabolism, redox and*

545   *photosynthesi- related proteins in both cultivars:* Eleven proteins (#1, 3, 8, 9, 10, 12, 18, 25, 27,

546   28, and 36) were more abundant in PI cultivar, with the majority involved in heat response and

547   photosynthesis. Among these, five proteins were in low abundance to WS when applied

548   independently. Twelve proteins (#3, 5, 7, 8, 9, 12, 14, 15, 22, 23, 24, and 31) were more abundant

549   to combined stress in R 95 cultivar. The majority were involved in metabolism, out of which, three

550   proteins were low in abundance to WS and two proteins to HS. Soybean cultivar PI 471938, which

551   exhibits a slow-wilting phenotype under water-deficit conditions, has proven to be a good genetic

552   resource in developing drought-resistant progeny (79).

553   *vi). MED37C as a candidate protein:* Our systems bioinformatics approaches studies

554   showed that more proteins were expressed in higher abundance to combined stresses in PI cultivar

555   and having more promotive interactions with other proteins, and in particular the proteins

556   associated with metabolism, and photosynthesis, leads to higher performance of the plant to the

557   multiple stresses. Protein MED37C (P26413), identified in our studies, was also observed to have

558   the good potential interacting partners in *Arabidopsis thaliana and* it has been shown to have two

559   promotive interactors in both cultivars. In the R95 cultivar, it has more inhibitory interactors (five),

560   suggesting that the effect of the protein interaction is more significant in PI than in R95 cultivar in

561   response to combined stresses. Therefore, we contemplate that the protein might also have

562   potential interacting partners in soybean. This candidate protein would be of potential interest as

563   it is a probable mediator of RNA polymerase transcription II associated with heat shock proteins

564   (80).

565   *vii). Co-relation of mRNA expression for selected stress-responsive proteins:* The protein

566   abundance levels as a result of differential expression of mRNA transcript levels can often be

567   correlated with their protein expression. Although most of the mRNA expression levels in this

568   study exhibited expression trend that were matching with corresponding protein abundance levels,

569   there is a mixed response in the expression and accumulation pattern of some mRNA and the

570   corresponding protein abundance profiles. In PI cultivar, expression of serine and heat shock

571   protein at both transcript level and protein abundance were corresponding with protein abundance

572   and were increased to all stress treatments. Whereas, the expressions of peroxidase, chalcone

573   flavone isomerase, ascorbate perodxidase, catalase, calireticulin, peroxiredoxin, and superoxide

574   dismutase transcripts were in contrast to their protein abundance. There is conflict as the protein

575   abundance is not correlated with the expression of corresponding transcript levels. In cultivar R

576   95, the expression levels of transcript follow their relative abundance in proteins. Broadly, catalase,

577   serine, chalcone flavone isomerase were up-regulated at transcript level and showed relatively high

578   abundance of proteins in response to the stress treatments, while calireticulin, peroxidase,

579   peroxiredoxin, ascorbate peroxidase, superoxide dismutase, and heat shock protein were low at

580   both transcript and protein levels. Reports indicate a poor correlation between mRNA and protein

581   abundances in the cell and depend on various biological and technical factors (81). The association

582   may not be as similar because the mRNA transcription is relatively lower than protein translation

583   (82). Protein levels are more conserved than mRNA levels, and their turnover is probably

584   influencing the correlation between mRNA and protein abundances to a higher degree (83).

585   *viii). Elevated activities in antioxidant enzymes in PI-373819 cultivar enhances the*

586   *tolerance to ROS production and builds homeostasis:* Antioxidant enzyme activities displayed

587   significantly higher levels in PI cultivar to all stress treatments, despite their activities low under

588   control conditions when compared with those of R95 cultivar. SOD is considered first-line defense

589   against toxic effects of elevated ROS and the increase in SOD in PI cultivar to all three stresses

590   would play a significant role in ROS scavenging in plants and is considered as the first line of

591   defense against the toxic effects of elevated ROS levels (84). SOD catalyzes the dismutation of

592   superoxide radicals to H_2O_2 and O_2. The increase of SOD activity might be the reason for enhanced

593   O_2 generation, as a result of electron leakage from the electron transport chains to molecular

594   oxygen (85). Water stress or combined WS+HS reportedly induce oxidative stress in plants (86).

595   POD is the primary enzyme and the increase in its activity detoxifies H_2O_2 in chloroplast and

596   cytosol during oxidative stress (87). Both POD and CAT constitute a main H_2O_2 scavenging

597   system during oxidative stress induced by WS or HS or both (88). Similar observations of higher

598   POD and CAT activities were reported in tolerant genotypes implicating their role in developing

599   resistance to one or more stresses when compared to the decrease in these enzyme levels in

600   susceptible genotypes (89).

601   APX activity and GR were elevated in PI cultivar to all stresses, while their levels GR has

602   not detected in R 95 cultivar when treated with combined stresses. Overexpression of APX seems

603   to play a key role in regulating of H_2O_2 levels in plant cells by preventing H_2O_2 from reaching the

604   nuclei from cytosol, inhibiting lipid peroxidation and protein oxidation and thereby making the

605   cultivar more tolerant (90). Increased APX indicates that the PI cultivar can restore the oxidation

606   levels and sustain the plant during the stress. GR keeps GSH/GSSG ratio favorable for ascorbic

607   acid reduction. The GR activity in PI cultivar preserved GSH/GSSH ratio due to lower incidence

608   of oxidative damage. In contrast, the absence of GR activity in R95 cultivar in response to

609   combined stress suggests impairment of GSH recycling due to enhanced ROS accumulation (91).

610   **Conclusions:**

611   We provides insights into proteome and enzyme responses to multiple stresses occurring

612   simultaneously in the field conditions of soybean. Our results showed that concurrent stresses

613   alter physiological, proteome and enzymes indifferent to individual stress. Cultivar PI-371938

614   maintained total biomass to all stresses, compared to R95-1705 cultivar. The degree of genetic

615   diversity was observed between two cultivars in their protein abundance when subjected to various

616   types of stresses. Several proteins involved in metabolism, response to heat and photosynthesis

617   have shown significant cross-tolerance mechanism. Cross-tolerance was evident in R95-1705

618   cultivar among heat responsive proteins, photosynthesis, metabolism and redox proteins that were

619   high in abundance to heat stress as well as to the combined heat and water stress in the PI-371938

620   cultivar suggesting the cultivar as relatively heat tolerant.   Both sets of proteins were adversely

621   affected when treated with heat stress alone. However, heat stress alone enhanced redox related

622   proteins in R95 cultivar.

623   Elevated activities in antioxidant enzymes, such as increased APX, indicate that the PI-

624   371938 cultivar has the ability to restore the oxidation levels and sustain the plant during the stress.

625   Proteins were elevated in high abundance to combined stress in PI-371938 demonstrated more

626   promotive interactions associated with metabolism, photosynthesis leading to continued resistance

627   to both types of stress. Protein MED37C, a probable mediator of RNA polymerase transcription II

628   yielded potential protein interactors partners in *Arabidopsis* and our studies documents the

629   significant impact of the protein in PI cultivar. Levels of protein expression and transcripts

630   correlate with the regulation at transcription and post transcription levels. Furthermore, the milder

631   stress on small scale can mitigate the detrimental effect of extreme conditions.

632   **Acknowledgements:**

633   RK, NM, VP acknowledges the financial support from National Science Foundation Grant number

634   1156900 to carry out analysis of this research. Ms. Tiffany Boyunt reviewed and assisted in

635   preparing graphs. Ms. Catlyn Eliason and Ms. Sara Bergin read and edited the MS. The soybean

636   lines were generously supplied by Dr. Thomas E. Carter, Jr., USDA-ARS Soybean & Nitrogen

637   Fixation Unit, Raleigh, NC and Dr. Pengyin Chen, University of Missouri, Columbia, MO.

638   **Author contributions**

639   KR performed the laboratory experiments and wrote the manuscript draft and executed the

640   research, NM carried out analysis of the 2D gel data; SS revised the manuscript, collected the

641   literature for data discussion; SL performed the field experiments and collected the plant material;

642   PS conducted interactome data analysis and edited the manuscript; MK conducted Mapman bin

643   analysis and edited the manuscript; KS carried out mathematical prediction of protein interactions

644   and edited the manuscript; MB prepared figures and edited the manuscript; SS and VI prepared

645   tables, and edited the manuscript; KRR conceived the project, executed, and supervised this

646   research.

647   **Conflict of interest:**

648   All authors of this manuscript declare that they have no conflict of interest.

649   **Declarations:**

650   The corresponding authors certify that all authors have seen and approved the final version of the

651   manuscript being submitted and warrant that the article is the authors' original work, hasn't

652   received prior publication and isn't under consideration for publication elsewhere.

653   **References:**

654   [1] Food and Agriculture Organization of the United Nations, Crop statistics reports for the world-
655   wide area harvested of Soybean. http://www.fao.org/faostat/en/#data/QC/ 2019 (accessed 18
656   January 2019).
657

[2] O. Özener, L. Yüksek, A.T. Ergenç, M. Özkan. Effects of soybean biodiesel on a DI diesel engine performance, emission and combustion characteristics. Fuel. 2014; 115:875-83.

[3] X.P. Xu, H. Liu, L. Tian, X.B. Dong, S.H. Shen, L.Q. Qu. Integrated and comparative proteomics of high-oil and high-protein soybean seeds. Food chemistry. 2015; 172:105-16.

[4] N.Z. Lupwayi, A.C. Kennedy, M. Chirwa. Grain legume impacts on soil biological processes in sub-Saharan Africa. African Journal of Plant Science. 2011; 5:1-7.

[5] E.V. Tairo, P.A. Ndakidemi. Yields and economic benefits of soybean (*Glycine max* L.) as affected by *Bradyrhizobium japonicum* inoculation and phosphorus supplementation. American Journal of Research Communication. 2013; 1:159-172.

[6] J.T. Spargo, M.A. Cavigelli, S.B. Mirsky, J.E. Maul, J.J. Meisinger. Mineralizable soil nitrogen and labile soil organic matter in diverse long-term cropping systems. Nutrient Cycling in Agroecosystems. 2011; 90:253-266.

[7] S. Mourtzinis, J.E. Specht, L.E. Lindsey, W.J. Wiebold, J. Ross, E.D. Nafziger, H.J. Kandel, N. Mueller, P.L. Devillez, F.J. Arriaga, S.P. Conley. Climate-induced reduction in US-wide soybean yields underpinned by region-and in-season-specific responses. Nature plants. 2015; 1:14026.

[8] S. Komatsu, N. Ahsan. Soybean proteomics and its application to functional analysis. Journal of proteomics. 2009; 72:325-336.

[9] H. Le Gall, F. Philippe, J.M. Domon, F. Gillet, J. Pelloux, C. Rayon. Cell wall metabolism in response to abiotic stress. Plants. 2015;4:112-66.

[10] V.V. Ergo, R. Lascano, C.R. Vega, R. Parola, C.S. Carrera. Heat and water stressed field-grown soybean: A multivariate study on the relationship between physiological-biochemical traits and yield. Environmental and Experimental Botany. 2018; 148:1-1.

[11] Y. Liu, J. Wu, N. Sun, C. Tu, X. Shi, H. Cheng, S. Liu, S. Li, Y. Wang, Y. Zheng, V.N. Uversky. Intrinsically disordered proteins as important players during desiccation stress of soybean radicles. Journal of Proteome Research. 2017; 16:2393-409.

[12] S. Iuchi, M. Kobayashi, T. Taji, M. Naramoto, M. Seki, T. Kato, S. Tabata, Y. Kakubari, K. Yamaguchi-Shinozaki, K. Shinozaki. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. The Plant Journal. 2001; 27:325-33.

[13] S.I. Zandalinas, R. Mittler, D. Balfagón, V. Arbona, A. Gómez-Cadenas. Plant adaptations to the combination of drought and high temperatures. *Physiologia plantarum*. 2018; 162:2-12.

[14] K. Jumrani, V.S. Bhatia. Impact of combined stress of high temperature and water deficit on growth and seed yield of soybean. Physiology and Molecular biology of Plants. 2018; 24:37-50.

704
705   [15] W.T. Pettigrew. Potassium influences on yield and quality production for maize, wheat,
706   soybean and cotton. *Physiologia plantarum.* 2018; 162:2-12.
707   [16] J.M. Thomas, P.V. Prasad, K.J. Boote, L.H. Allen Jr. Seed composition, seedling emergence
708   and early seedling vigour of red kidney bean seed produced at elevated temperature and carbon
709   dioxide. Journal of Agronomy and Crop Science. 2009 Apr;195:148-56.
710
711   [17] W. Shi, X. Yin, P.C. Struik, C. Solis, F. Xie, R.C. Schmidt, M. Huang, Y. Zou, C. Ye, S.K.
712   Jagadish. High day-and night-time temperatures affect grain growth dynamics in contrasting rice
713   genotypes. Journal of experimental botany. 2017; 68:5233-5245.
714
715   [18] C. Dürr, S. Brunel-Muguet, C. Girousse, A Larmure, C. Larré, A. Rolland-Sabaté, M.H.
716   Wagner. Changes in seed composition and germination of wheat (*Triticum aestivum*) and pea
717   (*Pisum sativum*) when exposed to high temperatures during grain filling and maturation. Crop
718   and Pasture Science. 2018; 69:374-86.
719
720   [19] C. Ren, K.D. Bilyeu, P.R. Beuselinck. Composition, vigor, and proteome of mature soybean
721   seeds developed under high temperature. Crop science. 2009; 49:1010-1022.
722
723   [20] C.S. Carrera, J.L. Dardanelli. Water deficit modulates the relationship between temperature
724   and unsaturated fatty acid profile in soybean seed oil. Crop Science. 2017; 57:3179-89.
725
726   [21] M. Djanaguiraman, P.V. Prasad, D.L. Boyle, W.T. Schapaugh. Soybean pollen anatomy,
727   viability and pod set under high temperature stress. Journal of Agronomy and Crop Science. 2013;
728   199:171-177.
729
730   [22] M. Haslbeck, E. Vierling. A first line of stress defense: Small heat shock proteins and
731   theirfunction in protein homeostasis. Journal of molecular biology. 2015; 427:1537-48.
732
733   [23] J. Qin, F. Gu, D. Liu, C. Yin, S. Zhao, H. Chen, J. Zhang, C. Yang, X. Zhan, M. Zhang.
734   Proteomic analysis of elite soybean Jidou17 and its parents using iTRAQ-based quantitative
735   approaches. Proteome science. 2013 Dec;11(1):12.
736
737   [24] P.P. Mohammadi, A. Moieni, S. Hiraga, S. Komatsu. Organ-specific proteomic analysis of
738   drought-stressed soybean seedlings. Journal of Proteomics. 2012; 75:1906-23.
739
740   [25] S. Kidokoro, K. Watanabe, T. Ohori, T. Moriwaki, K. Maruyama, J. Mizoi, N. Myint Phyu
741   Sin Htwe, Y. Fujita, S. Sekita, K. Shinozaki, K. Yamaguchi-Shinozaki. Soybean DREB 1/CBF-
742   type transcription factors function in heat and drought as well as cold stress-responsive gene
743   expression. The Plant Journal. 2015; 81:505-18.
744
745   [26] F. Zhao, D. Zhang, Y. Zhao, W. Wang, H. Yang, F. Tai, C. Li, X. Hu. The difference of
746   physiological and proteomic changes in maize leaves adaptation to drought, heat, and combined
747   both stresses. Frontiers in Plant Science. 2016; 7:1471.
748

[27] G.B. Bhaskara, T.N. Wen, T.T. Nguyen, P.E. Verslues. Protein phosphatase 2Cs and microtubule-associated stress protein 1 control microtubule stability, plant growth, and drought response. The Plant Cell. 2017; 29:169-91.

[28] K. Kosová, P. Vítámvás, I.T. Prášil, J. Renaut. Plant proteome changes under abiotic stress-contribution of proteomics studies to understanding plant stress response. Journal of Proteomics. 2011; 74:1301-22.

[29] S. Komatsu, R. Kuji, Y. Nanjo, S. Hiraga, K. Furukawa. Comprehensive analysis of endoplasmic reticulum-enriched fraction in root tips of soybean under flooding stress using proteomics techniques. Journal of Proteomics. 2012; 77:531-60.

[30] A. Ashoub, M. Baeumlisberger, M. Neupaertl, M. Karas, W. Brüggemann. Characterization of common and distinctive adjustments of wild barley leaf proteome under drought acclimation, heat stress and their combination. Plant molecular biology. 2015; 87:459-71.

[31] I. Rejeb, V. Pastor, B. Mauch-Mani. Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. Plants. 2014; 3:458-75.

[32] M.A. Hossain, Z.G. Li, T.S. Hoque, D.J. Burritt, M. Fujita, S. Munné-Bosch. Heat or cold priming-induced cross-tolerance to abiotic stresses in plants: key regulators and possible mechanisms. Protoplasma. 2018; 255:399-412.

[33] Ü. Niienments. Responses of forest trees to single and multiple environmental stresses from seedlings to mature plants: Past stress history, stress interactions, tolerance and acclimation, Forest Ecology and Management. 2010: 260:1623–1639.

[34] K.R. Reddy, J.J. Reddy, J.M. McKinion. Soil-Plant-Atmosphere-Research (SPAR) facility: A tool for plant research and modeling. Biotronics. 2001; 30:27-50.

[35] D. Zhao, K.R. Reddy, V.G. Kakani, J.J. Read, J.H. Sullivan. Growth and physiological responses of cotton (*Gossypium hirsutum* L.) to elevated carbon dioxide and ultraviolet-B radiation under controlled environmental conditions. Plant, Cell & Environment. 2003: 26:771-82.

[36] E.J. Hewitt. Sand and water culture methods used in the study of plant nutrition. Commenwealth Agricultural Bureaux. 1966.

[37] H.K. Lichtenthaler. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Methods in enzymology. Academic Press. 1987; 148:350-382.

[38] A.M. Saxton. A macro for converting mean separation output to letter groupings in Proc Mixed. Proceedings of the 23rd SAS Users Group International, 22-25 Mar 1998, Nashville. 1998:1243-6.

[39] R. Katam, S.M. Basha, P. Suravajhala, T. Pechan. Analysis of peanut leaf proteome. Journal of Proteome Research. 2010; 9:2236-54.

[40] M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976;72;248-254.

[41] R. Katam, K. Sakata, P. Suravajhala, T. Pechan, D.M. Kambiranda, K.S. Naik, B. Guo, S.M. Basha. Comparative leaf proteomics of drought-tolerant and-susceptible peanut in response to water stress. Journal of proteomics. 2016; 143:209-26.

[42] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. An International Journal. 1999; 20:3551-67.

[43] R. Katam, K. Chibanguza, L.M. Latinwo, D. Smith. Proteome biomarkers in xylem reveal pierce's disease tolerance in grape. Journal of Proteomics & Bioinformatics. 2015; 8:217.

[44] B. Usadel, A. Nagel, O. Thimm, H. Redestig, O.E. Blaesing, N. Palacios-Rojas, J. Selbig, J. Hannemann, M.C. Piques, D. Steinhauser, W.R. Scheible. Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of coresponding genes, and comparison with known responses. Plant Physiology. 2005 Jul 1;138:1195-204.

[45] N. Tanaka, S. Mitsui, H. Nobori, K. Yanagi, S. Komatsu. Expression and function of proteins during development of the basal region in rice seedlings. Mol. Cell. Proteomics 2005, 274, 796–808.

[46] D. Warde-Farley, S.L. Donaldson, O. Comes, K. Zuberi, R. Badrawi, P. Chao, M. Franz, C. Grouios, F. Kazi, C.T. Lopes, A. Maitland. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic acids research. 2010; 38: W214-20.

[47] H.K. Vasanthaiah, K.V. Ravishankar, G.K. Mukunda. Mango. In:Fruits and Nuts 2007 (pp. 303-323). Springer, Berlin, Heidelberg.

[48] A. Untergasser, H. Nijveen, X. Rao, T. Bisseling, R. Geurts, J.A. Leunissen. Primer3Plus, an enhanced web interface to Primer3. Nucleic acids research. 2007;35: W71-4.

[49] C.N. Giannopolitis and S.K. Ries. Superoxide dismutase: I. Occurrence in higher plants. Plant Physiology. 1977; 59(2): 309-314.

[50] M. Kar and D. Mishra. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiology. 1976; 57(2): 315-319.

[51] I. Cakmak and H. Marschner. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiology. 1992; 98(4): 1222-1227.

840　[52] Y. Nakano, K. Asada. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in
841　spinach chloroplasts. Plant and Cell Physiology. 1981;22:867-80.

842　[53] C.L. Sgherri, F. Navari-Izzo. Sunflower seedlings subjected to increasing water deficit stress:
843　oxidative stress and defence mechanisms. *Physiologia Plantarum*. 1995; 93:25-30.

844

845　[54] P. Pandey, V. Ramegowda, M. Senthil-Kumar. Shared and unique responses of plants to
846　multiple individual stresses and stress combinations: Physiological and molecular mechanisms.
847　Frontiers in Plant Science. 2015; 6 (723): 1-14.

848

849　[55] Y. Bai, C. Kissoudis, Z. Yan, R.G.F. Visser, G. van der Linden. Plant behavior under
850　combined stress: tomato responses to combined salinity and pathogen stress. The Plant Journal.
851　2018; 93:781-793.

852

853　[56] E.S. Tozzi, H.M. Easlon, J.H. Richards. Interactive effects of water, light and heat stress on
854　photosynthesis in Fremont cottonwood. Plant, cell & environment. 2013; 36:1423-1434.

855　[57] M.A. Parry, J. Flexas, H. Medrano. Prospects for crop production under drought: Research
856　priorities and future directions. Annals of Applied Biology. 2005; 147:211-26.

857

858　[58] S.K. Singh, V.G. Kakani, G.K. Surabhi, K.R. Reddy. Cowpea (*Vigna unguiculata* [L.] Walp.)
859　genotypes response to multiple abiotic stresses. Journal of Photochemistry and Photobiology B:
860　Biology. 2010;100:135-146.

861

862　[59] J. Zhang, J. Liu, C. Yang, S. Du, W. Yang. Photosynthetic performance of soybean plants to
863　water deficit under high and low light intensity. South African Journal of Botany. 2016; 279-287.

864

865　[60] R. Zhou, X. Yu, C.O. Ottosen, E. Rosenqvist, L. Zhao, Y. Wang, Z. Wu. Drought stress had
866　a predominant effect over heat stress on three tomato cultivars subjected to combined stress. BMC
867　Plant Biology. 2017; 17: 24.

868

869

870

871　[61] J.A. Rollins, E. Habte, S.E. Templer, T. Colby, J. Schmidt, M. Von Korff. Leaf proteome
872　alterations in the context of physiological and morphological responses to drought and heat stress
873　in barley (*Hordeum vulgare* L.). Journal of Experimental Botany. 2013; 64:3201-3212.

874

875　[62] V.M. Rodríguez, P. Soengas, V. Alonso-Villaverde, T. Sotelo, M.E. Cartea, P. Velasco. Effect
876　of temperature stress on the early vegetative development of *Brassica oleracea* L. BMC plant
877　biology.2015; 15: 145.

878

879　[63] M. Weng, L. Cui, F. Liu, M. Zhang, L. Shan, S. Yang, X. Deng. Effects of Drought Stress on
880　Antioxidant Enzymes in Seedlings of Different Wheat Genotypes. Pakistan Journal of Botany.
881　2015; 47: 49-56.

882

883　[64] Y. Ahmad, F.M. Boisvert, E. Lundberg, M. Uhlen, A.I. Lamond. Systematic analysis of
884　protein pools, isoforms, and modifications affecting turnover and subcellular localization. 2012.
885　Mol Cell Proteomics, 2012; 11(3): M111.013680.

886
887 [65] M. Stastna, J. E. Van Eyk. Analysis of protein isoforms: can we do it better? Proteomics,
888 2012; 12(0): 2937–2948.
889
890 [66] C. Boldner, T. Skroppa, O. Johnsen, A. Polle. Freezing tolerance in two Norway spruce (*Picea*
891 *abies* [L] Karst) progenies is physiologically correlated with drought tolerance. 2005. Journal of
892 Plant Physiology 162: 549-558.
893
894 [67] T. J. A. Bruce, M. C. Matthes, J. A. Napier, J. A. Pickett. Stressful memories of plants:
895 evidence and possible mechanisms. Plant Sci. 2007; 173, 603–608.
896
897 [68] N. Ahsan, T. Donnart, M.Z. Nouri, S. Komatsu. Tissue-specific defense and thermo-adaptive
898 mechanisms of soybean seedlings under heat stress revealed by proteomic approach. Journal of
899 Proteome Research. 2010; 9:4189-204.
900
901 [69] N.K. Sarkar, P. Kundnani, A. Grover. Functional analysis of Hsp70 superfamily proteins of
902 rice (Oryza sativa). Cell stress and Chaperones. 2013; 18:427-437.
903
904 [70] H. Huang, I.M. Møller, S.Q. Song. Proteomics of desiccation tolerance during development
905 and germination of maize embryos. Journal of proteomics. 2012; 75: 1247-1262.
906
907 [71] D. Ray, A. Ghosh, S.B. Mustafi, S. Raha. Plant stress response: Hsp70 in the spotlight. In
908 Heat Shock Proteins and Plants. 2016: Springer, Cham:123-147.
909
910 [72] T.M. Hildebrandt, A.N. Nesi, W.L. Araújo, H.P. Braun. Amino acid catabolism in plants.
911 Molecular Plant. 2015; 8: 1563-15.
912
913 [73] X. Wang, X. Cai, C. Xu, Q. Wang, S. Dai. Drought-Responsive Mechanisms in Plant Leaves
914 Revealed by Proteomics. 2016; 17:1706.
915
916 [74] S. Kadam, K. Singh, S. Shukla, S. Goel, P. Vikram, V. Pawar, N. Singh. Genomic associations
917 for drought tolerance on the short arm of wheat chromosome 4B. Functional & Integrative
918 Genomics. 2012; 12:447-464.
919
920 [75] R. Waditee-Sirisattha, H. Kageyama, Y. Tanaka, M. Fukaya, T. Takabe. Overexpression of
921 halophilic serine hydroxymethyltransferase in freshwater cyanobacterium Synechococcus
922 elongatus PCC7942 results in increased enzyme activities of serine biosynthetic pathways and
923 enhanced salinity tolerance. Archives of Microbiology. 2017; 199: 29-35.
924
925 [76] P. Mishra, A. Jain, T. Takabe, Y. Tanaka, M. Negi, N. Singh, N. Jain, V. Mishra, R. Maniraj,
926 S.L. Krishnamurthy, R. Sreevathsa, N.K. Singh, V. Rai. Heterologous expression of serine
927 hydroxymethyltransferase-3 from rice confers tolerance to salinity stress in E. coli and
928 *Arabidopsis*. 2019. Frontiers in Plant Science, 10 (217).
929
930 [77] D. Hughes. Elongation Factors: Translation. *Reference Module in Life Sciences*. (2017).
931

[78] K. Kosová, P. Vítámvás, M. Urban, M. Klíma, A Roy, I Prášil. Biological networks underlying abiotic stress tolerance in temperate crops-a proteomic perspective. International Journal of Molecular Sciences. 2015;16: 20913-20942.

[79] W. Sadok, Matthew E. Gilbert, M. Aown S. Raza, and Thomas R. Sinclair. Basis of slow-wilting phenotype in soybean PI 471938 Crop Sci. 2012; 52:1261–1269.

[80] B. Huang, and C. Xu. Identification and characterization of proteins associated with plant tolerance to heat stress. J Integr Plant Biol. 2008; 50(10):1230-1237.

[81] G. Wu, L. Nie, and W. Zhang. Integrative analyses of posttranscriptional regulation in the yeast *Saccharomyces cerevisiae* using transcriptomic and proteomic data. Curr. Microbiol. 2008; 57, 18–22.

[82] I. Kosti , N. Jain, D. Aran, A.J. Butte, and M. Sirota. Cross-tissue analysis of gene and protein expression in normal and cancer tissues. Sci Rep. 2016; 6:24799.

[83] Doherty, M.K., Hammond, D.E., Clague, M.J., Gaskell, S.J. and Beynon, R.J. (2009) Turnover of the human proteome: determination of protein intracellular stability by dynamic SILAC. J. Proteome Res. 8, 104–112.

[84] S.S. Gill, N. Tuteja. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 2010; 48(12):909-930.

[85] E. Yıldıztugay, A.H. Sekmen, I. Turkan M. Kucukoduk. Elucidation of physiological and biochemical mechanisms of an endemic halophyte *Centaurea tuzgoluensis* under salt stress. Plant Physiology and Biochemistry. 2011; 49(8):816-824.

[86] S.I. Zandalinas, D. Balfagón, V. Arbona, A. Gómez-Cadenas. Modulation of antioxidant defense system is associated with combined drought and heat stress tolerance in citrus. Frontiers in Plant Science. 2017; 8:953.

[87] N. Etemadi, M.H. Sheikh-Mohammadi, A. Nikbakht, M.R. Sabzalian, M. Pessarakli. Influence of trinexapac-ethyl in improving drought resistance of wheatgrass and tall fescue. *Acta Physiologiae Plantarum. 2015;* 37(3):53.

[88] N.A. Akram, F. Shafiq, M. Ashraf. Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. Frontiers in Plant Science. 2017; 8: 613.

[89] N. Ara, K. Nakkanong, W. Lv, J. Yang, Z. Hu, M. Zhang. Antioxidant enzymatic activities and gene expression associated with heat tolerance in the stems and roots of two cucurbit species ("*Cucurbita maxima"* and *"Cucurbita moschata*") and their interspecific inbred line "*Maxchata*". Int. J. Mol. Sci. 2013; 14:24008-24028.

[90] K. Apel, H. Hirt. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Ann. Rev. Plant Biol. 2004; 55:373-399.

[

91] C. H. Foyer and G. Noctor, Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 2011. 155: 2-18.

Table 1 Temperature (T, °C) and irrigation (IRR: well-watered, WW; water stressed, WS) effects either alone or in combinations on soil water content (SWC, m^3 m^{-3}), leaf relative water content (LRWC, %), chlorophyll concentration (Chl, µg cm^{-2}), photosynthetic rate (P_{net}, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), chlorophyll fluorescence (Fv′/Fm′), internal CO_2 concentration (C_i, µmol CO_2 mol^{-1}), transpiration (Tr, mmol H_2O m^{-2} s^{-1}), and total biomass (TBM, g $plant^{-1}$) of two soybean cultivars (C) between 48 and 50 days after emergence. Treatments (T and IRR) were initiated 34 days after emergence. The data are the mean of the three-six individuals (the mean of ten individuals for total biomass). Analysis of variance (*ANOVA*) between T, IRR, and C are given.

Cultivar	Temperature	Irrigation		SWC	LRWC	Chl	P_{net}	g_s	Fv′/Fm′	C_i	Tr
PI 373819	28/20	WW		0.0855bc	83.1a	37.4c	28.82a	1.399b	0.557a	353ab	16.11c
	28/20	WS		0.0045d	71.1cd	39.6bc	1.95de	0.055c	0.435b	323abc	1.66d
	38/30	WW		0.0899ab	76.5bc	39.5bc	27.72ab	1.866a	0.543a	362a	27.51b
	38/30	WS		0.0013d	64.2e	39.6bc	0.67e	0.022c	0.325c	319b	1.28d
R95 – 1705	28/20	WW		0.0962a	80.6ab	40.0abc	24.79c	1.308b	0.544a	350ab	16.81c
	28/20	WS		0.0033d	70.1d	39.5bc	3.28d	0.054c	0.396b	287c	1.72d
	38/30	WW		0.0829c	79.5ab	43.8a	25.86bc	1.810a	0.551a	360a	32.08a
	38/30	WS		0.0013d	63.7e	41.5ab	2.60de	0.015c	0.331c	193d	0.84d
	ANOVA	*ANOVA*									
		C		ns	ns	*	ns	ns	ns	***	ns
		T		*	**	ns	ns	*	***	*	***
		IRR		***	***	ns	***	***	***	***	***
		C	T	**	ns	ns	ns	ns	ns	*	ns
		C	IRR	ns	ns	ns	**	ns	ns	***	*
		T	IRR	ns	ns	ns	ns	**	***	**	***
		C T IRR		**	ns	ns	ns	ns	ns	*	ns

Significant at *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; and ns = non-significant ($P > 0.05$). Within columns for each experiment, means followed by same letters are not significantly different at α = 0.05

997

a Spot #	b Accession	c Phytozome ID	Description	dMr (Da) /epI-fTheo	dMr (Da) /dpI gExp	Molecular function	Biological Process	Mowse Score	hCov. %
1. Response to Heat									
*1	P26413	Glyma17g08020	Heat shock Protein 70	73.9/5.20	66/4.55	ATPase activity, Heat shock protein binding	Stress-related, Protein refolding	876	50
*3	P26413	Glyma17g08020	Heat shock Protein 70	73.9/5.21	66/4.63	ATPase activity, Heat shock protein binding	Stress-related, Protein refolding	876	50
*27	Q39818	Glyma12g01580	Heat shock Protein 22* (mitochondrial)	22.0/6.24	24/6.8	Calvin cycle, Rubisco interacting	Stress-related	395	52
*28	Q39818	Glyma12g01580	Heat shock Protein 22* (mitochondrial)	22.0/6.24	26.6/5.8	Calvin cycle, Rubisco interacting	Stress-related	395	52
*31	P04795	Glyma14g06910	Heat shock protein*17.6 kda class 1	17.6/5.69	15/5.65	Protein self-association, Unfolded protein binding	Response to heat, Stress-related protein complex oligomerization,	255	66
*32	P04795	Glyma14g06910	Heat shock protein*17.6 kda class 1	17.6/5.69	15/5.65	Protein self-association, Unfolded protein binding	Stress-related	255	66
34	B4X941	VIGUN *Vigna unguiculata*	17.7 kda class 1 heat shock protein	17.8/6.85	17/6.2	NA	Stress-related	978	82

2. Protein Re-folding

2	P08824	Glyma12g08310	Chaperonin subunit alpha 60 kda	61.7/5.23	63/4.4	ATP binding, Calvin cycle, Rubisco interacting	Protein refolding	312	27
6	P02581	Glyma05g09290	Actin	41.9/5.31	51/5.4	ATP binding	Signal transduction	217	19
11	A0A762	Glyma10g28890	Calreticulin	48.2/4.4	50/7.3	Unfolded protein binding	Signal transduction	150	36

3. Oidation-Reduction process

5	B0M1A4	Glyma06g02040	Catalase	55.2/6.5	55/5.35	Catalase activity, Heme binding, Metal ion binding	Redox, Responsive to H2O2	65	29
14	Q9ZT38	Glyma04g41990	Alcohol dehydrogenase	41.1/6.32	40/5.1	Oxidoreductase activity, Zin ion binding	Oxidation, reduction	1340	78
19	Q43758	Glyma11g15680	Ascorbate peroxidase	27.1/5.5	34/6.2	Heme binding, L-ascorbat peroxidase activity, Metal ion binding	Redox, Cellular response to oxidative stress	328	57
26	C6SZ56	Glyma19g42890	Superoxide dismutase (SOD)	21.5/6.28	21/6.4	Metal ion binding, Superoxide dismutase activity	Redox	NA	NA
29	B3GV28	Glyma07g09240	Peroxiredoxin	17.4/5.4	14/4.3	Oxidoreductase activity	Cell Redox hemostasis	266	41

4. Metabolism

4	A8IKE5	Glyma02g04320	Alanine aminotransferase 2	52.1/6.92	54/5.9	Photorespiration, Pyridoxal phosphate bonding, Transaminase activity	Biosynthetic process	226	23
7	O82560	Glyma14g39420	Glutamine synthetase	47.9/6.4	47/5.1	ATP binding, Glutamate-ammonia ligase activity, Identical protein binding	Metabolism, Glutamine biosynthetic process	192	37
12	C6ZJZ0	Glyma18g150000	Serine hydroxy methyl transferase 5"	57.1/8.13	59/7.65	Glycine hydroxymethyl transerase activity	Metabolism	165	24
13	O23963	Glyma05g02670	Translation elongation factor	52.3/6.21	42/4.9	GTPase activity, GTP binding, Translation elongation factor activity	Metabolism	303	27
15	E5RPJ6	Glyma05g27260	Pyruvate dehydrogenase	38.9/5.70	39/5.6	Catalytic activity	TCA	1443	36
18	O81278	Glyma05g01010	NAD dependent malate dehydrogenase	43.9/6.47	33/5.5	L-malate dehydrogenase activity	TCA process, Malate metabolic process	185	24

20	Q38IW8	Glyma15g04290	Triosephosphate isomerase	33.3/6.3	28/6.25	Triose-phosphate isomerase activity	Metabolism, Glycolytic process	107	14
21	Q93XE6	Glyma20g38560	Chalcone flavon isomerase 1A	23.3/6.23	26/6.15	Flavonoids, chalcone isomerase activity	Secondary metabolism	325	54
*22	O22443	Glyma09g02590	Peroxidase*	38.6/6.0	41/6.7	Heme binding, Peroxidase activity, Metal ion binding	H2O2 catabolic process	1463	37
*23	O22443	Glyma09g02591	Peroxidase*	39.1/8.45	40/7.1	Peroxidase activity	Metabolism	1463	37
24	Q9XJ23	Glyma12g01000	Acid phosphatase	29.2/8.75	29/7.1	Acid phosphatase activity, Gluconeogenesis	Metabolism	134	39
33	Q9SWA8	Glyma12g04701	Glycine-rich RNA binding protein	16.7/5.5	14/6.20	RNA binding, Transcription regulation	Metabolism	424	55
37	Q8GV24	Glyma07g13710	Nucleoside diphosphate kinase	16.5/6.3	15/6.55	ATP binding, Nucleoside diphosphate kinase activity	Metabolism	1067	62
39	O82561	Glyma14g39421	Glutamine synthetase	47.9/6.4	47/5.1	Nitrogen metabolism, Glutamine synthetase	Metabolism	192	37

5. Photosynthesis

8	D4N5G3	Glyma11g34230	Rubisco activase	14.6/6.8	50/61	ATPbinding, Ribulose-1,5-bisphosphate carboxylase/oxygenase activator activity	Photosynthesis, Calvin cycle	327	68
9	Q6RIB7	Glyma19g37520	Enolase	47.9/5.49	53/6.15	Acetyl-CoA C-acyltransferase activity, Magnesium ion binding, Phosphopyruvate hydratase activity	Glycolytic process	208	34
16	I1JJ05	Glyma02g45190	Oxygen-evolving enhancer protein 2	27.7/8.27	29/4.7	Calcium ion binding	Photosynthesis	NA	NA
17	Q2IOH4	Glyma06g18110	Glyceraldehyde 3-phosphate dehydrogenase	36.8/6.72	37/5.65	NAD binding	Metabolism, Glucose meta glycolytic process	1052	70
25	Q39831	Glyma05g25810	Chlorophyll A/B-Binding Protein	27.9/5.29	22/4.9	Chlorophyll binding	Photosynthesis, Light harvesting	68	19
30	Q39832	Glyma19g06370	Ribulose bisphophate carboxylase small chain 1	14.6/6.8	12/4.3	Calvin cycle activity, Ribulose-bisphosphate carboxylase activity	Photosynthesis, carbon fixation, Photorespiration	327	68

| 38 | Q6RUF6 | Glyma14g01470 | Fructose bisphosphate aldolase | 38.6/7.1 | 13.5/7.1 | Calvin cycle, Fructose-bisphosphate aldolase activity | Photosynthesis, Glycolytic process | 587 | 46 |

6. Others

10	Q6RIB8	Other	5-hydroxy tryptamine receptor 4	47.9/5.50	53/6.5	G protein-coupled serotonin receptor activity	Morphogenesis	208	34
35	P10743	Glyma08g21410	Stem 31 kda glycoprotein precursor	29.4/6.7	15/6.2	Nutrient reservoir activity, Acid and other phosphatases	Seed storage	720	65
36	Q9ZTZ2	Glyma17g16620	Late embryogenesis abundant protein	49.5/7.1	12/6.1	Embryo development ending in seed dormancy	Seed developemnt	789	40

[a] Spot number as given on the 2-D gel image in Figure 2

[b] Protein identification number as in Uniprot/NCBI database

[c] Protein identification number as in Soybean phytozyme database

[d] Protein molecular weight

[c] pI value

[e] Theoretical value

[f] Experimental Value

[g] Identified peptide coverage

*Protein resolved in multiple spots on 2DE

998
999
1000