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# Physiological, Proteomic, and Biochemical analysis Reveal Possible Cross-Tolerance in metabolism and heat response proteins in response to Heat and Water Stress in Soybean

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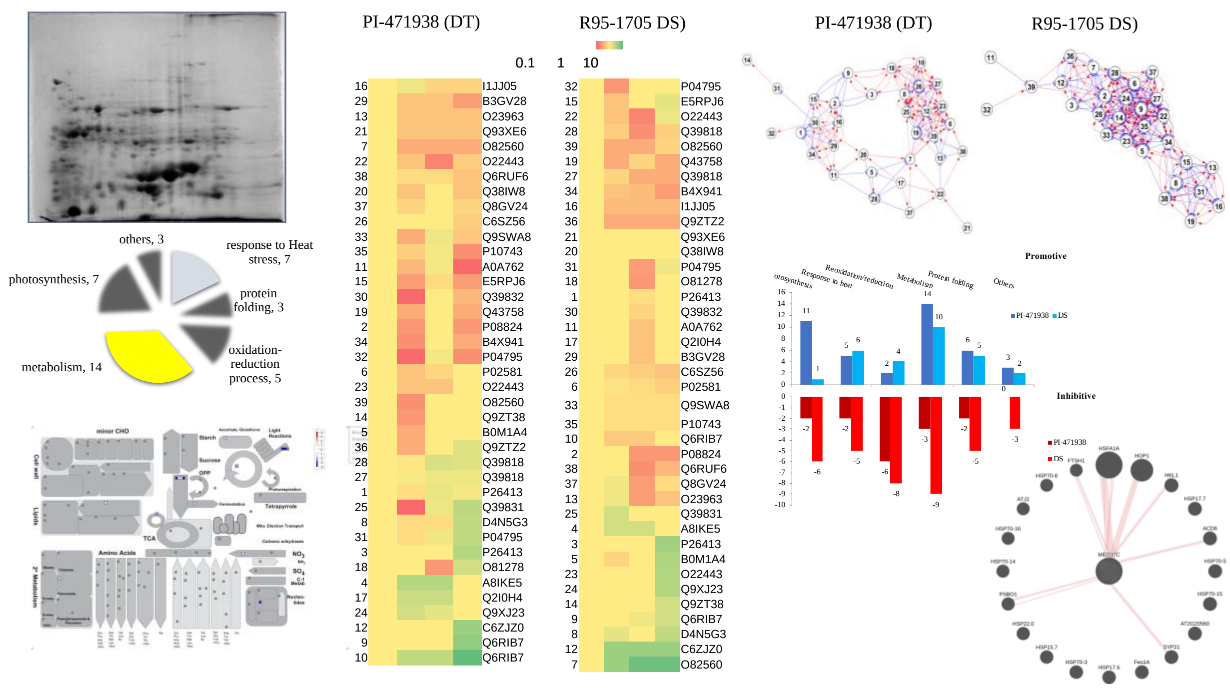
**Running Title:** Soybean Leaf Proteome responses to multiple abiotic stresses

**Graphical Abstract:**



**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 destruction of photosynthetic apparatus, macromolecules within cells and the onset of complex signaling cascades. Changes in the physiological characteristics, proteome, and certain metabolites were investigated on molecular and cellular functions were studied in two soybean cultivars that were 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. 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, majority involved in metabolism, response to heat and photosynthesis have shown significant cross-tolerance mechanism. Functional analysis revealed a majority of heat responsive-proteins were more abundant during HS and combined stress (WS+HS) while these proteins were low to WS in cultivar PI 471938 while 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, whereas, In cultivar R 95, the expression levels of transcript follow their relative abundance in proteins. Protein MED37C, a probable mediator of RNA polymerase transcription II yielded potential protein interactors partners in *Arabidopsis* and our studies documents the significant impact of the 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 plant during the stress. Our study

hypothesizes the plant's development of cross-stress tolerance and will help foster the ongoing ventures in genetic modifications in stress tolerance.

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

## Introduction:

*Glycine max* (L.) Merr (soybean) is a legume that provides a significant source of proteins and fatty acids in both human and animal diets. It is an important legume crop grown for its combustion fuel, cooking oil, and protein with over 121.5 million hectares worldwide (FAO, 2019). It is the largest source of feed protein in the world and second largest source of food oil (Ozener et al., 2014; Xu et al., 2015). Soybean crop plays a major role in contributing to soil fertility as they are naturally capable of fixing atmospheric nitrogen and the root exudates of some legumes can solubilize phosphorus and other insoluble calcium-bound phosphorus compounds (Lupwayi et al, 2011; Tairo et al, 2013). The presence of legumes ameliorate the soil quality by encouraging microbial activity, especially around its rhizosphere, by contributing to organic matter restoration, and play a role in disease prevention and pest control (Spargo et al, 2011). Soybean has been widely adapted and cultivated across the climatic zones of the world.

Soybean has also been an important model crop for $C_3$ annual plants, because of its strong response to climate change. For example, a 17% decrease in yield for 1°C rise in temperature have been observed (Mourtzinis et al, 2015). The overall production of soybean is severely limited by several abiotic factors that include flooding, drought, salinity, and acidity (Komatsu and Ahsan, 2009). Due to its various developmental stages, the abiotic factors strongly impact the plant's growth. Therefore, it is essential to protect crop yields from higher and more frequent episodes of extremely high temperatures and drought both in current and future climates. Water or heat stress is involved in cell dehydration and affects various metabolic functions in plants. Water stress (WS) is one of the most debilitating factors of soybean crop with dehydration in plants leading to a disruption in the water potential gradients, loss of turgor pressure, denaturation of proteins, which also leads to a lack in the unraveling of cellular membranes (Gall et al, 2015; Ergo et al, 2018).

Another devastating effect of dehydration is desiccation, in which the protoplasmic "free water" is lost and the cell is required to survive on the water bound within the cell matrix (Liu et al, 2017). It has also been found that plants respond to dehydration by alternating levels of protein synthesis and protein degradation, with a recent evidence suggesting that there is a direct correlation between the accumulation of proteins synthesized by dehydration stress and the plant's physiological adaptations to water stress (Iuchi et al., 2001; Zandalinas et al, 2018). In addition, when soybeans have encountered water stress during the reproductive stage, owing to a lack of plasticity to recover at this stage, showed much detrimental decrease in seed yield and its attributes, compared to the plants grown under irrigated condition (Jumrani et al, 2018).

Heat stress (HS) in the form of high temperatures during flowering, is a cardinal factor limiting seed count in many crops, including soybean (Pettigrew, 2008; Thomas et al, 2009; Shi et al, 2017; Dürr et al, 2018). High temperatures are found in many southern regions of the United States during the germination season of soybean plants. Temperature above 30°C affect germination by decreasing the seed vigor of the soybean. As a result, the levels of stachyose and phytic acid in soybean seeds are decreased, which leads to difficulties in membrane biogenesis and germination (Ren et al, 2009). While it has been reported that exposure to elevated temperatures encourages oil and protein production in the soybean plants, extreme temperatures result in changes to the seed oil concentration and in the ratios of singular fatty acids to total fatty acids in the soybean oil (Carrera et al, 2017). High temperatures can also lead to desiccation of the seeds and cause abnormal exine structure during microsporogenesis resulting in pollen malformation (Djanaguiraman et al, 2013). Heat stress tolerance is controlled by adjustments in the membrane structure and function, tissue water content, protein composition, lipid activity, and by primary and secondary metabolites (Haslbeck et al, 2015). Several reports showed changes in molecules in response to water and temperature stresses at transcription and protein levels that affect photosynthetic efficiency, and the activity of nitrate reductase. The levels of soluble proteins in soybean cultivars were directly correlated with the leaf rate of photosynthesis (Qin et al. 2013).

In response to drought, proteins involved in photosynthesis, signaling pathways, and reactive oxygen species detoxification were severely impacted (Mohammadi et al., 2012). Both heat and water stress in soybean induced overexpression of *DREB1* family genes, several dehydrins, and LEA genes resulting in their over representation among up-regulated genes in soybean plants under heat and drought stresses (Kidokoro et al, 2015). In soybean most of the differentially abundant proteins were related to photosynthesis, ATP synthesis, and protein biosynthesis (Zhao et al, 2016). Gene expression of different proteins is directly influenced by WS treatment. For instance, when the cellular and biochemical components that are triggered by drought result in the activation or suppression of certain genes, and consequently the proteins involved in cell division, cell growth, and cell differentiation (Bhaskara et al, 2017). In response to heat stress, overexpression of enzymes involved in homeostasis, as well as the accumulation of various chaperone proteins, especially heat-shock proteins, were observed (Kosova et. al., 2011). The rhizomes of soybean plants have shown that dehydration has a negative effect on the levels of proteins that are responsible for protein transport and storage, ATP synthesis, metabolism, and signal transduction (Komatsu et. al, 2012). Therefore, proteomic analysis under multiple stresses will lead to better determination of the molecular pathways and the molecules associated with the complex cross-tolerance.

Earlier studies described relative abundance in leaf protein composition under water or heat stress in various crop plants (Ashoub et al, 2015; Zhao et al, 2016). However, the interactive effects and complex cross-tolerance mechanisms associated with physiological, biochemical, and proteome changes to heat and water stress are not well understood. As plants undergo a combination of multiple stresses in the field condition, they trigger defense mechanisms and cooperative systems, which under multiple stresses display cross tolerance with each other to increase the plants' immune efficiency (Rejeb et al, 2014). Cross-tolerance is a phenomenon in plants that makes them become more tolerant to second stress after imposing under first stress, such as induction of stress memory after the stress (Hossain et al, 2018). Simultaneous occurrence of more than one stress can have both positive and negative impacts on the plants performance and adaptation (Niienments 2010). Therefore, determining the key regulators that take part in orchestrated responses to concurrent stresses provide better understanding of tolerance mechanisms. The objective of present study was to determine the effect of water stress, heat stress, and combined stresses on the regulation of leaf proteins in two contrast soybean cultivars.

## Materials and methods:

### *2.1: Experimental facility*

The study was conducted in four sunlit, Soil-Plant-Atmosphere-Research (SPAR) plant growth chambers located at Mississippi Agriculture and Forestry Experiment Station, Mississippi State, MS, USA (www.spar.msstate.edu). Chamber air temperature, $CO_2$, and soil watering were controlled to provide automatic acquisition and storage of the data from the units, monitoring SPAR environments every 10 seconds (Reddy et al., 2001; Zhao et al., 2003). The high temperature, 38/30° and drought stress at optimum and high temperatures were imposed by withholding irrigation until the soil water content reached 96% (-30 M Pa) of the control (-1.5 M Pa). Leaf samples were collected from the plants for proteome analysis.

*2.2: Plant growth and treatments*

Two contrasting soybean cultivars - PI 471938 (slow wilting and high yielding; Cultivar#2) and R95 – 1705 (high in protein concentration and moderate yield potential- Cultivar#1) were used in the experiment. Detailed experimental procedure outline is shown in Figure 1. Soybean seeds for both cultivars were planted in three rows. Plants were thinned to 10 plants per row 12 days after emergence (DAE) and irrigated three times a day with half-strength Hoagland's nutrient solution (Hewitt, 1966). The air temperature of 28/20°C (day/night) was maintained until the beginning of the treatments (30 DAE). Thereafter, four treatments consisting of two levels of each factor, temperature (28/20° and 38/30°C) and irrigation (well-watered-WW, and water stress-WS) were imposed until the harvests (57 DAE). The control treatments consisted of 28/20°C and WW. The WS was imposed gradually as follows: no irrigation (30-31 DAE), watered 40% of the control (32-39 DAE), and no irrigation (40-50 DAE). The high temperature, (38/30°) and drought stress at optimum and high temperatures were imposed by withholding irrigation until the soil water content reached 96% (-30 M Pa) of the control (-1.5 M Pa).

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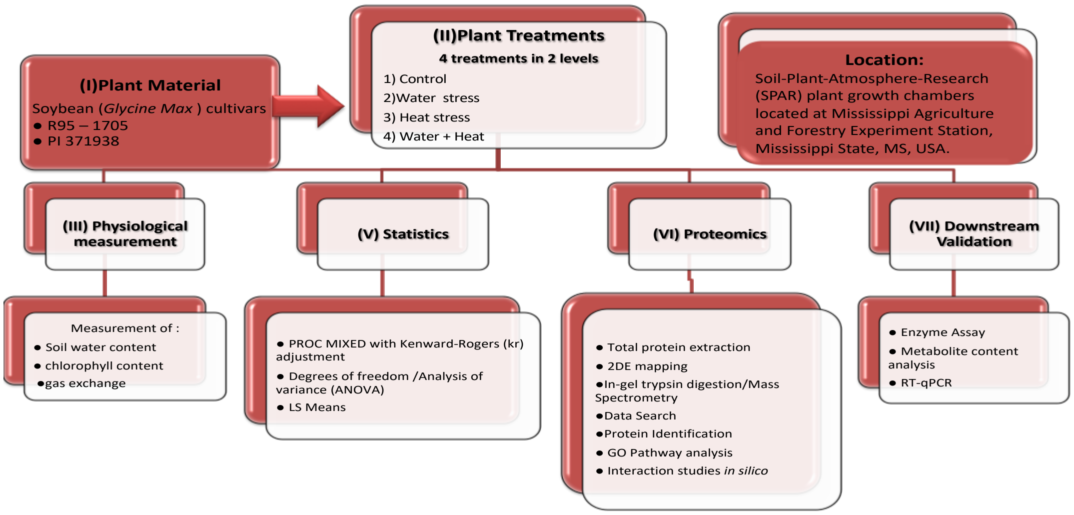


Figure 1: Experimental outline, deciphering the analysis of physiological and biochemical changes in response to stress in soybean

*2.3: Physiological measurements*

*a. Soil water content:* The soil water content (SWC) was measured with a soil moisture probe (Delta-T Devices, Burwell, UK). Leaves of soybean plants were detached to measure the leaf's fresh, turgid and dry weights and the relative leaf water content was determined as follows: LWC = (fresh weight – dry weight)/ (turgid weight – dry weight).

*b. Chlorophyll content:* Total chlorophyll was extracted by placing five 0.38 $cm^{-2}$ leaf disks for each row in a vial containing dimethyl sulfoxide (5ml) and incubated in dark for 24h. Thereafter, the absorbance of the supernatant was measured using a UV/VIS spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The total chlorophyll was estimated and expressed on leaf area basis (Lichtenthaler1987).

*c. Measurement of gas exchange and chlorophyll fluorescence:* Gas exchange and chlorophyll fluorescence parameters were measured simultaneously using a Li-Cor 6400 Photosynthesis system (Li-Cor Inc., Lincoln, Nebraska, USA). The values of $P_{net}$, Fv′/Fm′, stomatal conductance ($g_s$), intercellular ($C_i$) $CO_2$ concentration, and transpiration were automatically computed from the instruments software.

*2.4. Statistical analysis*

Statistical analyses were performed using SAS (SAS Enterprise Guide, 4.2, SAS Institute Inc., NC, USA). PROC MIXED with Kenward-Rogers (kr) adjustment of degrees of freedom was used for analysis of variance (ANOVA) to test the effect of treatments and cultivars, and their interactions on the plant and soil water status, chlorophyll concentrations, gas exchange and fluorescence parameters, and total biomass. Treatments (temperature and irrigation) and cultivars were considered as the fixed effect, and individual measurements / rows were the random effects. The treatment comparisons were conducted by a least square means (LSMEANS) procedure (at $\alpha$=0.05) and the letter grouping was obtained using pdmix800 macro (Saxton, 1998).

*2.5. Total protein extraction of soybean leaf*:

The uppermost fully expanded leaves were detached and collected into liquid nitrogen. Leaves were collected from six plants of the same cultivar then frozen in liquid nitrogen and stored at -80 C prior to protein extraction. Protein was extracted following the modified procedure (Katam et al., 2010). Briefly, frozen powder (6 g) was vortexed in 20 ml of 50 mM Tris HCl (pH 7.5) containing 2 M thiourea, 7 M urea, 2% Triton X-100, 1% DTT and 4% PVPP. The suspension was centrifuged at 5000 rpm and the protein was precipitated with TCA (15%). The protein pellets were washed twice in cold acetone (-20° C) and centrifuged for 15 min at 13000 rpm. Final pellets were resuspended in IEF rehydration solution [7 M urea, 2% CHAPS (w/v), 2 M thiourea, 0.2% DTT (w/v)] to measure the protein concentration (Bradford, 1976).

*2.6. 2-DE Protein Mapping:*

An aliquot (300 µg in 100 µl) of the protein extract was loaded on to the tube gels and isoelectric focusing (IEF) was performed as described previously (Katam et al., 2016). Tube gels were then loaded on a slab gel and the proteins were resolved by electrophoresis.

*2.7. Gel image and Statistical analysis:*

Gels were scanned using a Gel Image system (Bio Rad, Hercules, CA). The Analysis Set derived from three replicated gels of matched spots that were present on all the gels and the three replicated gels, were analyzed using PD Quest (version 8.0.1). A one-way analysis of variance (ANOVA) was conducted to compare the mean protein spot densities and test if there was any difference in the protein spot abundance among the treatments and two cultivars studies. The differentially expressed spots (with P-values $<0.05$) showing significant differences were chosen for further analysis. Protein spots were manually excised from gels following in-gel digestion, and MALDI/TOF mass spectrometry.

*2.8. In-Gel Trypsin Digestion:*

The in-gel digestion mixture was a disulfide bond reduction. The resulting peptide mix was desalted with $C_{18}$ Zip Tips (Millipore), and 0.7 µl of the eluate and 5mg/ml matrix (α-cyano-4-hydroxycinnamic acid) was spotted on the ABI 01-192-6-AB MALDI plate (Applied Biosystems, Foster City, CA).

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

Mass spectra were collected on the ABI 4700 Proteomics Analyzer (Applied Biosystems) MALDI/TOF mass spectrometer (MS) and protein identification was performed using the automated result dependent analysis of ABI GPS Explorer software, version 3.5 (Applied Biosystems). Data was analyzed as Peptide Mass Fingerprinting (PMF), and protein identifications done by searching against the database using the MOWSE algorithm (Perkins et al., 1999). Both

MS and MS/MS data were matched against Phytozyme the soybean taxonomic database. Only the proteins with a total score of confidence interval (C. I) % > 95% were considered as positive identities.

*2.10. Gene Ontology (GO) Annotation:*

The identified proteins were mapped to Universal Protein Resource (UniProt KB) to assess their functional analysis as previously described (Katam et al., 2015). The accessions were queried using batch Entrez to retrieve several sequences that mapped to different proteins. The annotations and accession numbers were retrieved using the GO Retriever tool and were grouped into different levels. Protein sequences were searched against gene ontology tools and Target P program to derive functional classification, cellular localization and further validated using MapMan bin codes (Usadel et al., 2005).

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

Time course expression data was used to estimate the interaction among the proteins in both cultivars. Protein-protein interactions were estimated by temporal expression profiling utilizing an *S-system* differential equation [R1] as previously described (Tanaka et al., 2005). Furthermore, from the association studies, 39 proteins were interolog mapped to *Arabidopsis* database using GeneMania (Warde-Farley et al. 2010).

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

Total RNA was isolated using a modified CTAB-based protocol for RT-qPCR (Vasanthaiah et al 2007). A NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and agarose gel electrophoresis were used to test RNA quality and quantity. Total RNA from each sample was reverse-transcribed using an iScript cDNA Synthesis kit (Bio-

Rad, Hercules, CA, USA). Gene-specific primers (Supplementary Table I) were designed with the Primer Premier 5.0 (Untergasser et al., 2007).

*2.13. Enzyme assay:*

Superoxide dismutase, Catalase and peroxidase activities were measured as described in literature (Giannopolitis and Ries 1977; Kar and Mishra 1976; Cakmak and Marschner 1992). The leaf tissue was homogenized in 50 mM of $KPO_4$ buffer (pH 7.0) for ascorbate peroxidase (APX). The supernatant was used for APX assay from the decrease in absorbance at 290 nm (Nakano and Asada 1981). Absorbance of non-enzymatic oxidation of ascorbate by $H_2O_2$ was used as control. The reaction was measured by the decrease in absorbance at 340 nm. Glutathione reductase (GR) was determined using the protocol (Sgherri et al., 1995) by measuring the reduction of GSSG by NADPH and measuring the absorbance at 340 nm.

**Results:**

Two soybean cultivars were used in this study to determine the changes in physiological, molecular, metabolite and enzyme activities in leaf tissues subjected to water stress or heat stress and combination of both.

**1. Physiological measurements:**

*Soil water content (SWC), chlorophyll content, measurement of gas exchange and chlorophyll fluorescence:* Water stress caused a severe decrease in soil water content (SWC) and relative leaf water content (LWC) leading to a decrease in photosynthetic rate ($P_{net}$), stomatal conductance ($g_s$) transpiration (Tr) and total biomass (TBM) in both cultivars. In plants grown at 28/20°C under well water (WW) conditions, the plant responses to water stress (WS) was more severe than high temperature (38/30°C) with the exception of the stomatal conductance ($g_s$) and the transpiration (Tr) which increased 33-39 and 71-91% at high temperature, respectively

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(Table1). Among the physiological parameters, the C □ T □ IRR and the C □ T and interactions were significant ($P \leq 0.01$) for SWC and $C_i$. The T □ IRR interaction was significant ($P \leq 0.01$) 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 Tr. The treatments (T and IRR) significantly ($P \leq 0.001$) affected all the parameters except a few incidents of chlorophyll concentration, $P_{net}$, and TBM. Chlorophyll content was increased in response to combined water and temperature stress in the cultivar PI 471938 while it was reduced in the R-95 cultivar. With the exception of chlorophyll concentration in both cultivars, water stress in combination with high temperature showed the greater reduction in these parameters. Water stress caused 14% reduction in leaf water content in both cultivars, while it was further reduced by 21% when both stresses were imposed. Compared to the control (well-watered), water stress severely reduced $P_{net}$ (>86%), $g_s$ (>95%), and transpiration (>89%), either alone or in combination with high temperature in both cultivars. However, high temperature alone increased both $g_s$ (>33%) and transpiration (>70%) in both cultivars. R95–1705 showed greater decrease in TBM (50%) than 471938 (38%) under water stress at high temperature.

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

*i) 2-D Gel Electrophoresis and identification of relative abundant stress responsive proteins*: The 2-DE analytical gels of leaf proteins revealed that most of the proteins had a molecular weight ($M_r$) between 10 and 66 kDa, and pI between 4.3 and 7.9, a pattern typically observed in most of the leaf tissues (Figure 2). PD Quest digital image analysis and visual spot-by-spot validation of the match derived from 2-DE gels when carried out at a sensitivity reading of 5.0 revealed over 200 proteins in both the cultivars (Supplementary Table II). Cultivar PI 373819 showed 25% decrease to WS, an 8% increase to HS and 10% decrease to both WS+HS in total proteins; while cultivar R-95 showed a decrease by 6.6% to WS, 33% to HS, and a 10% to

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both WS+HS.

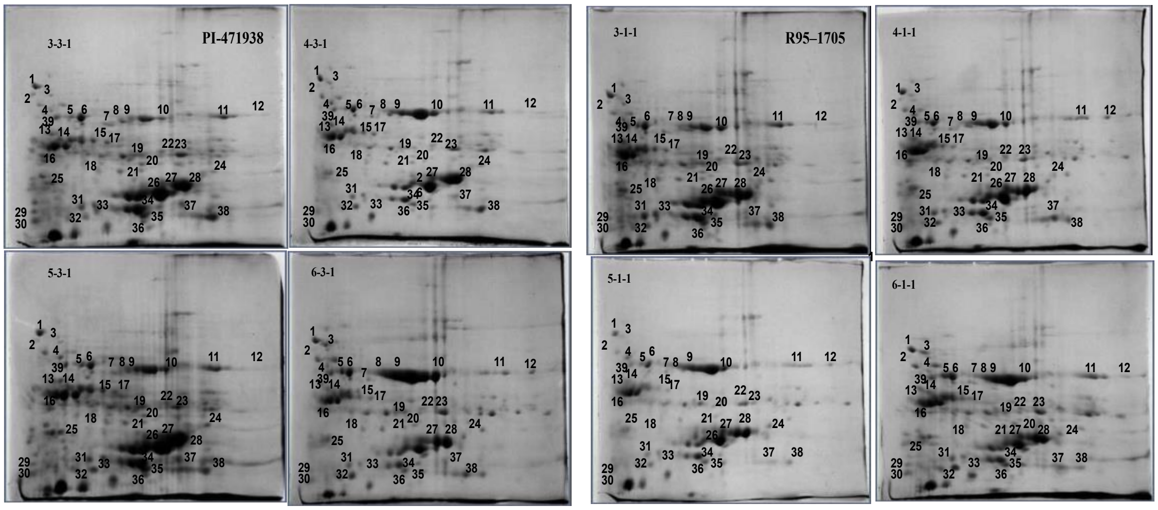


**Figure 2:** Changes in leaf protein abundance (protein spots with numbers) to water (WS), heat (HS) and combined stresses (WS+HS) in soybean cultivars

Comparative analysis of protein quantification profiles of both cultivars was carried out to identify the relative abundance of proteins in both cultivars to both stress conditions. Its considered >1.5-fold increase or <1.5 fold decrease as threshold level to determine the protein abundance based on the co-relation coefficient (r N 0.95), following the procedure published by Su et al 2013. Comparative analysis of protein profiles revealed 39 protein spots, showing quantitative variation following water stress or heat stress that satisfies the 95% confidence interval (Table 2).

*ii) Identification and functional classification of relative abundant Stress responsive proteins:* All of these 39 protein spots accounted for 31 non-redundant proteins, with 8 protein spots (4 proteins) showing similar protein accessions detected in multiple locations with differences in their isoelectric points and/or molecular weights on 2-DE gel. Relative protein abundance to various treatments between two cultivars and close up view of selected protein spots were is shown as in Supplementary Figure I and II. To determine the functional categories related to stress responses, the proteins were classified into five major functional categories including metabolism (14), response to heat (7), photosynthesis (7), redox process (5), protein re-folding (3)

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and others (3) (Figure 3a). Over half of the proteins belonged to metabolism, response to heat and photosynthesis. The molecular function of each protein is shown in Figure 3b.

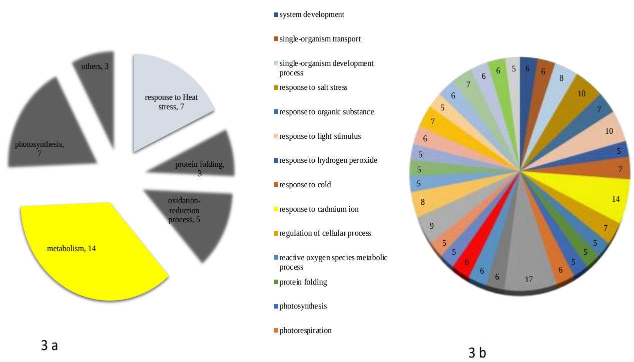


**Figure 3:** Gene ontology of stress responsive proteins: (A) Biological, (B) Molecular functions

Some of the biological functions of the proteins in control and treatments of both cultivars were validated using MapMan (Figure 4, Supplementary Figure III). The protein data was also analyzed to determine their association with individual organelles. The results predicted that they are localized in chloroplast, mitochondria, and others (Figure 5).

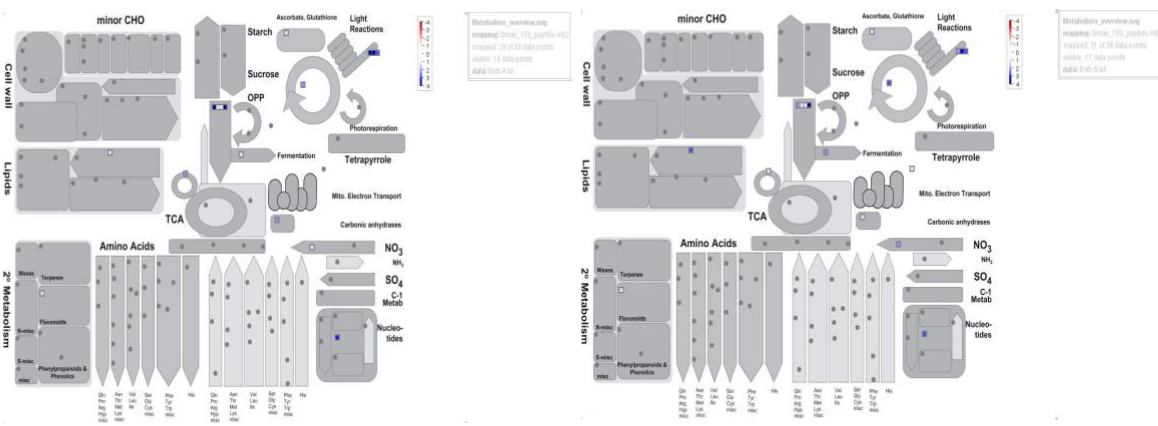


**Figure 4:** Phytozyme confirmations of protein identities using MapMan

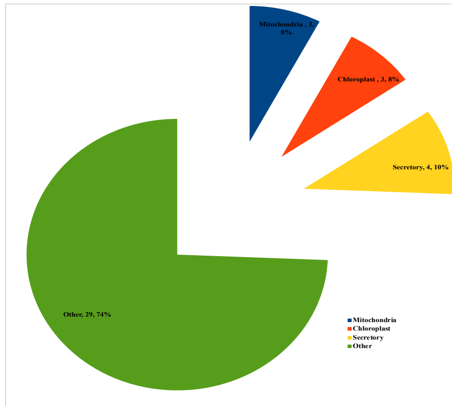


**Figure: 5**: Sub-cellular location prediction using TargetP

*iii). Clustering and dynamics of stress responsive proteins:* Expression analysis of the thirty-nine relatively abundant proteins were carried and analyzed (Figure 6). Four clusters of protein expression were recognized in both cultivars.

In cultivar PI 471938 (slow wilting and high yielding) I clustered proteins that were increased in abundance to either water stress (WS) or heat stress (HS) or the combination of water and heat stress (WS+HS) (Figure 6a). Out of eight proteins (# 3, 4, 9, 10, 12, 17, 24, and 27) the majority of them are involved in metabolism (3) photosynthesis (2), and response to heat (2) and other (1). Among the proteins in Cluster I, four proteins showed high abundance to more than two stresses (#4 10, 17 and 27) of which, two proteins (#10 and 17) showed increase in abundance to all three types of stresses. Cluster II includes 19 proteins (#2, 5, 6, 7, 13, 14, 15, 19, 20, 21, 22, 23, 26, 29, 30, 34, 37, 38, 39) that are in low abundance in response to at least one or more of the three stress types. The majority of these proteins involve in metabolism (9), oxidation-reduction reactions (5). Fifteen proteins in cluster II were low in abundance to two or more stresses. Seven of them (#7, 13, 15, 21, 22, 29, 38) were low in abundance to all three types of stresses; the majority of them were involved in metabolism. None of the proteins fall under the category of Cluster III in PI cultivar. Cluster IV includes 12 proteins (#1, 8, 11, 16, 18, 25, 28, 31, 32, 33, 35, 36) exhibiting mixed responses to each stress type/s, with the majority of them were involved in response to heat (3), and photosynthesis (3).

In cultivar R-95 (high in protein concentration and moderate yield potential), cluster I displayed a group of 10 proteins increased in their abundance to at least one type of stress, either to WS, or to HS, or to the combined WS+HS (Figure 6b). Those proteins (#3, 4, 7, 8, 9, 12, 14, 23, 24, and 25) are mainly involved in metabolism (5), following photosynthesis (3), response to heat (1), and oxidation-reduction reactions (1). Among these proteins five of them showed resistance to two or more stresses involved in metabolism and photosynthesis. Four proteins showed high abundance to WS, and three to HS. Protein # 7 and 12 involved in metabolism were not present in control but expressed in different quantities to all stresses studied. Cluster II includes 22 proteins that are in low abundance to one or more of the stresses (either WS, or HS or WS+HS) treatment (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 those involved in response to heat (5), photosynthesis (4), metabolism (4), protein refolding (3), oxidation reduction (3), and others (3). Among these, sixteen proteins showed reduction in abundance to two or more stresses, majorly involved in response to heat. ten proteins showed reduction in abundance to all three stresses. Cluster III proteins (spot # 20, and 21), which are involved in metabolism showed no response to water stress, heat stress and combined WS+HS stresses. Cluster IV includes six proteins (Spot #5, 13, 15, 22, 31 and 37), mostly involved in metabolism (4) which showed mixed response to the stresses, low abundance to one type and high to another type stress.

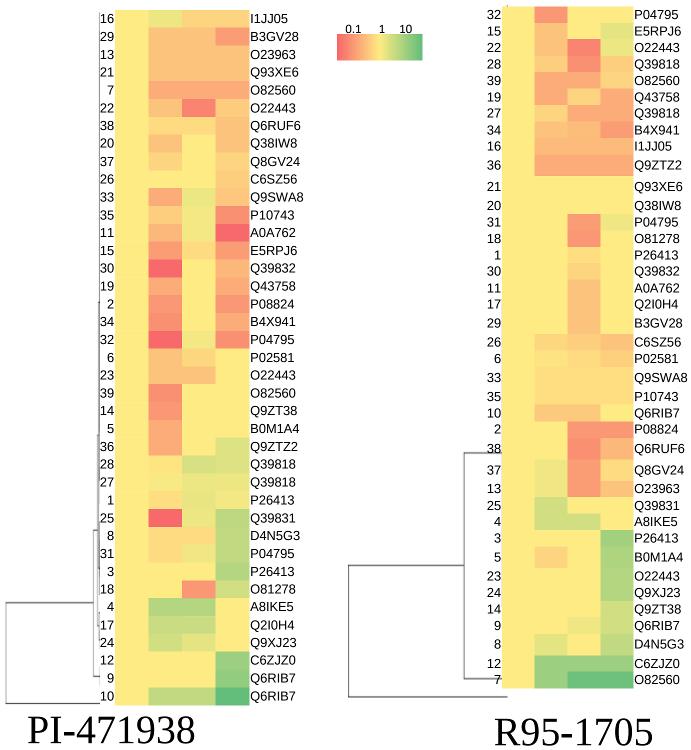


**Figure 6:** Relative abundance profile of 39 stress responsive proteins (SRPs) in a) PI and b) R-95 cultivars as depicted in hierarchical cluster.

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 Protein-protein interaction networks of differentially expressed proteins to abiotic stresses:* Relative abundance of stress responsive proteins was 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 to both WS+HS stress over a 3-week duration. Thirty-two proteins have shown interaction in the PI cultivar, while 29 have interacted in R95 cultivar. In PI cultivar, 95 promotive, and 61 inhibitive interactions were observed, while 113 promotive and 139 inhibitive interactions were observed in R95 cultivar (Figure 7; Supplementary Table III).

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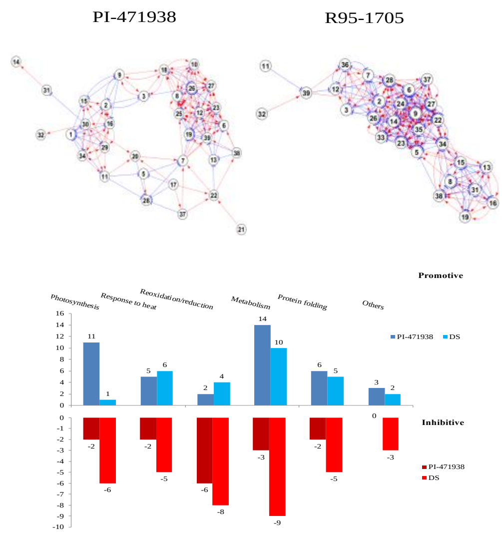



**Figure 7:** Protein interactions among SRPs.

A: Protein interactions in PI cultivar showing more interactions compared R95. The number of interacting proteins involved in various biological functions is shown in C. More proteins involved in protein synthesis and metabolism are associated with interactions in PI cultivar than R95. D. The estimated interactions were evaluated based on a goodness-of-fit calculated from "multiple-correlation coefficient" ($R_2$) between an expression profile and simulated profile of a protein positioned on the downstream side of an interaction. The interactions showing an r2 value (coefficient of determination) >0.9 were considered as candidate interactions. We calculated the $R_2$ corresponding to the interaction that a protein regulated the expression of another protein based on a modified version of the S-system differential equation. Spot numbers are the same as in Table II.

To further validate the interaction, the proteins that showed high abundance to combined WS+HS were further analyzed to determine the frequency of promotive and inhibitory interactions in both cultivars. PI 471938 showed high abundance of 11 proteins belonging to heat resistance (3), oxidation-reduction (2), metabolism (5), and photosynthesis (2) to combined WS+HS (Supplementary Table IV). These proteins displayed 41 promotive, and 15 inhibitive interactions. Among the promotive interactions, the majority were involved in metabolism, response to heat and photosynthesis. Cultivar R95–1705 showed high abundance in 8 proteins involved in resistance to heat (4), photosynthesis (2), metabolism (1), and other (1) (Supplementary Table V). These proteins displayed 28 promotive and 36 inhibitive interactions. Among the promotive interactions, majority were involved in metabolism.

When the 39 SRPs were subjected for interolog mapping and protein interaction analysis, heat shock protein 70 (MED37C) showing potential interactions with stress related proteins indicate that these orthologs in *Arabidopsis thaliana* genome have good potential interacting partners (Figure 8).

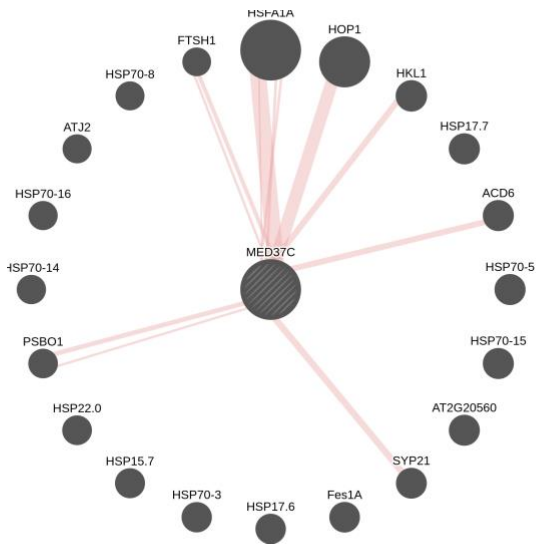
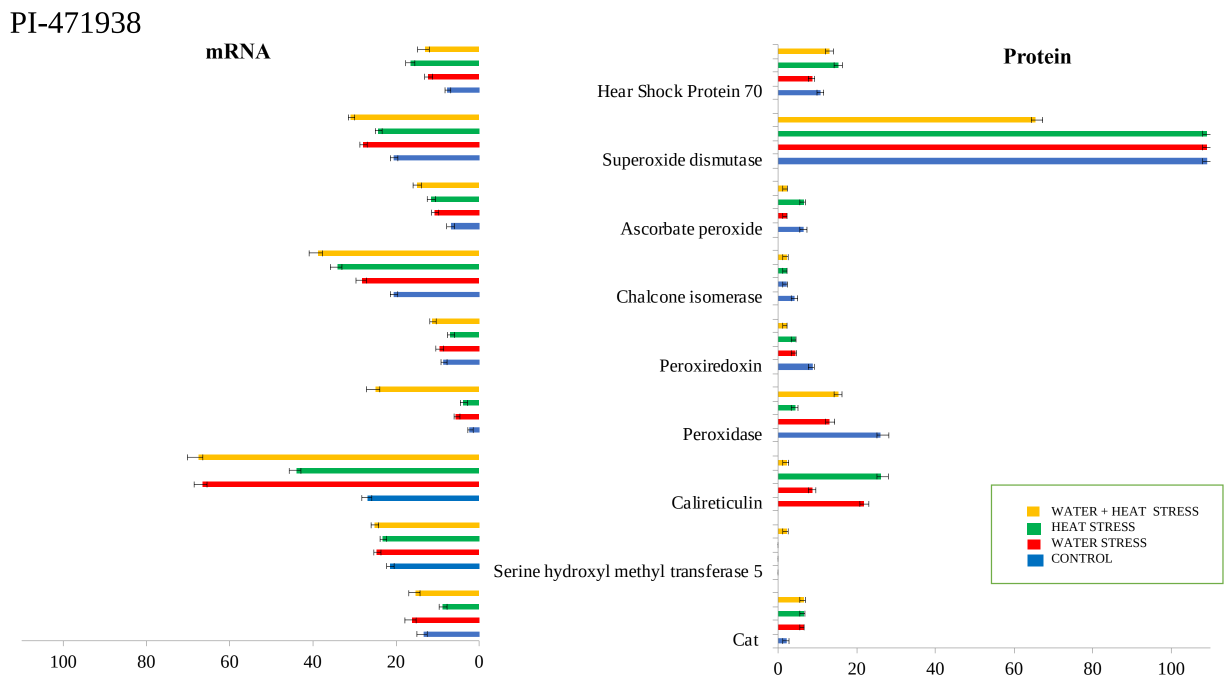


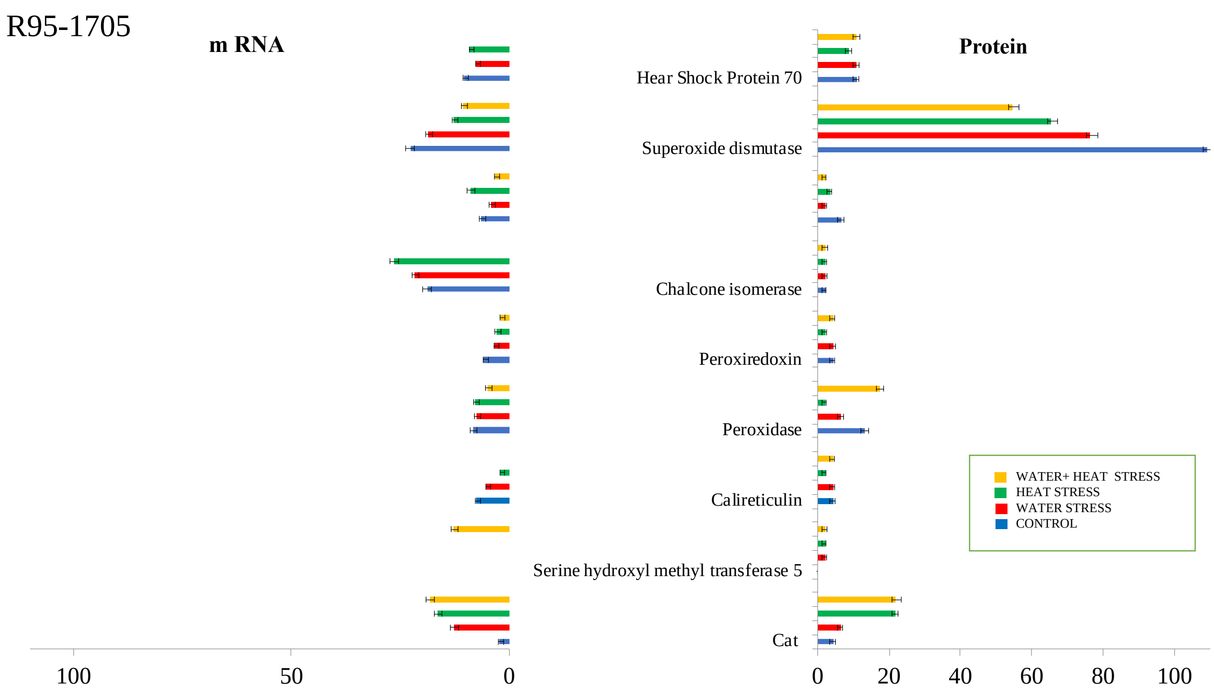

**Figure 8:** Protein-protein interaction map of the gene MED37C

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

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

Selected stress responsive proteins (SRP) were selected for transcriptional level expression analysis. Leaf samples from control and stressed plants were collected for mRNA extraction and then analyzed for RT qPCR. Results showed that, in cultivar PI 471938, the mRNA levels of ascorbate peroxide, chalcone flavone isomerase, serine hydroxymethyl transferase 5, calreticulin (CA), peroxidase, heat shock protein 70, superoxide dismutase showed up regulation to all stress treatments, while catalase and peroxiredoxin showed reduced levels to HS treatment (Figure 9a). In R95-1705 cultivar, majority of the transcripts showed mixed responses (Figure 9b). Catalase and serine hydroxymethyl transferase 5 showed up regulation of transcripts while calreticulin (CA), heat shock protein 70, peroxidase, peroxiredoxin and superoxide dismutase showed down regulation to all stress treatments. Expression of ascorbate peroxide increased to HS while reduced to WS, and WS+HS treatments. Chalcone flavone isomerase levels were increased to WS and HS while this transcript did not express at combined stress treatment.



 doi:10.20944/preprints201910.0307.v1

**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 were 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 cultivars, with an exception that GR was not detected in R 95 cultivar when treated with combined stresses.

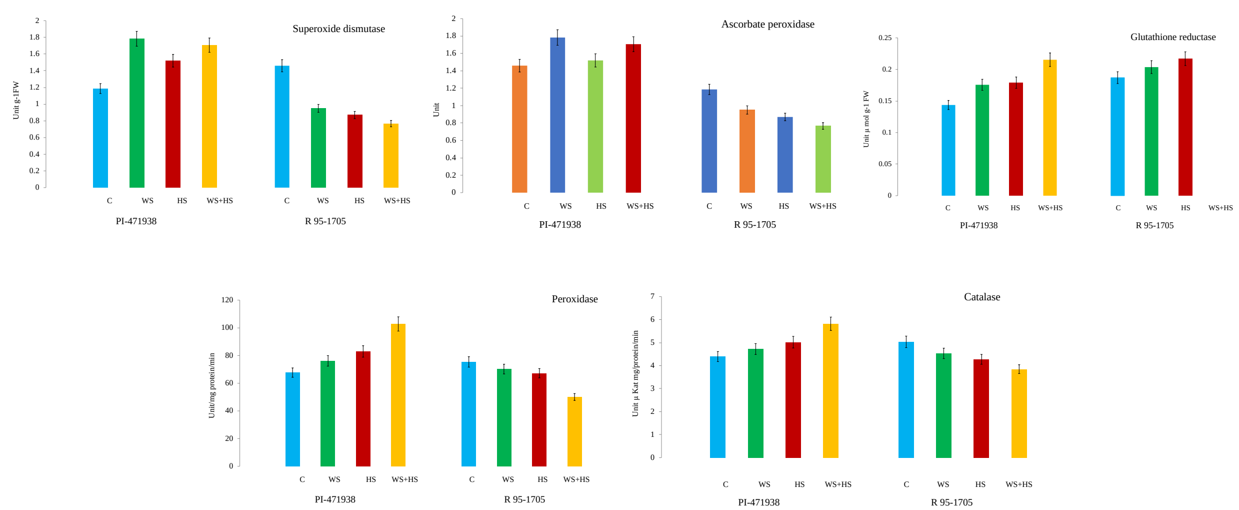


**Figure 10:** Enzymatic activities under water stress, heat stress and the combined stress. (The activity is measured in terms of fold change over the control; C: Control; WS: Water stress; HS: Heat stress; WS+HS: Water and heat stress).

**Discussion:**

Plants responses to concurrent stresses often occurring in the field, are very exclusive, when compared to individual stress treatments, and will display cross-tolerance to better adapt to those stresses (Pandey et al, 2015; Bai et al, 2018). We report the result of our studies on two contrast soybean cultivars to determine the physiological and molecular mechanism for cross-tolerance between multiple stresses.

*i) Greater reduction in Total biomass to stress treatments in R95-1705 cultivar:* The observed reduction in photosynthetic rate ($P_{net}$), stomatal conductance ($g_s$), transpiration (Tr) and total biomass (TBM) under water stress alone or in combination with high temperatures was closely associated with decrease in soil and plant water status. In both cultivars, the impact of water stress was several folds greater than the impact of high temperature, when these stresses are applied independently. WS, HS, WS+HS reduced stomatal conductance which is caused by leaf water potential via transpiration rate alterations in both cultivars. Photosynthesis is among the primary processes affected by WS+HS (Tozzi et al., 2013). No significant reduction in photosynthesis was observed during HS alone, whereas the consequences of water deficiency due to WS, or WS+HS have greater impact on altering photosynthetic machinery in both cultivars. Both stomatal conductance ($g_s$) and transpiration (Tr) was severely reduced under water stress, accompanied with reduction in the soil and plant water status. Plants partially close stomata to reduce the net transpiration under water stress, which might lead to the observed severe reduction in the photosynthesis ($P_{net}$) (Parry et al., 2005). However, the combined effect of two or more stress factors including high temperature have been reported to be more deleterious than the effect of a single stress factor on plant growth (Singh et al., 2010). Earlier studies showed reduction in photosynthesis and dry matter productions due to water stress and high temperature in soybean (Zhang et al., 2016; Zhou et al., 2017; Jumrani et al. 2018). Compared to the control, despite the lesser reduction in $P_{net}$ and leaf water content, cultivar R95–1705 showed greater decrease in the total biomass than in PI 471938.

Changes in proteins and anti-oxidative enzymes implicate the morphological and physiological adaptation in plants to stress (Rollins et al., 2013; Rodriguez et al., 2015). Hence, the proteomic studies were carried out to evaluate relative abundance of proteins and correlate with corresponding enzyme activities using two contrasting soybean cultivars in response to drought stress. Furthermore, we studied the effect of individual and combined stresses to water and heat stress and to determine the molecular mechanism for cross-tolerance between multiple stresses. Genetic variation was observed for responses to two stresses. PI cultivar was affected to WS displaying a 25% reduction in proteins compared to R95 cultivar, whereas, R95 exhibited a greater reduction in response to HS in proteins. Reduction in proteins was reported in wheat heat sensitive cultivar compared to tolerant wheat (Weng et al., 2015). However, when combination of WS+HS was administered, a similar reduction rate in the number of proteins was observed in both cultivars. Identification of protein isoforms with the same accession numbers spotted at multiple locations may be present due to alternative splicing, polymorphism, and post-translation modifications and they add to the proteome complexity (Ahmad et al, 2012). Often the protein isoforms differ in their cellular concentration and can be used as biomarkers (Stastna et al, 2012).

*ii) Dynamics of stress responsive proteins indicates PI-*471938 *cultivar, a heat tolerant:* Cluster I proteins that showed high abundance to one or more stresses, in contrast to cluster II proteins which showed low abundance to one or more stresses are represented in almost identical numbers in both cultivars, and the majority are metabolism proteins. Among cluster II proteins, greater reduction in abundance of heat response proteins and photosynthesis related proteins were observed in the R 95-1705 cultivar. In R 95-1705, eleven proteins were reduced in abundance to all three types of stresses, in particular, affecting more heat response proteins followed by redox proteins. In PI cultivar, seven proteins showed reduced abundance in all three stress types, primarily involving in metabolism and redox proteins. Cluster III proteins were all involved in metabolism (spot # 18, 20, and 21) showed no response to either water, or, heat, or combined stresses in R 95, while they were low in abundance in PI cultivar.

Cluster IV proteins that displayed mixed responses included twelve proteins from PI cultivar and six proteins from R-95 cultivar. In PI cultivar, ten proteins showed potential cross-tolerance, five with water and the combined stresses, and five with heat stress and the combined stress, the majority of them are heat response proteins. Heat response proteins that were overly abundant to combined stress in PI were also more abundant to HS, while two of them were low abundant to WS, suggesting that, the cultivar is more responsive and tolerant to HS and to combined HS+WS stress than WS alone.

The stress memory to one stress may prevent damages accruing from other stresses (Blodner et al., 2005). Plants use stress memory to stabilize the performance when exposed to infrequent environmental changes and increase resilience. Our studies revealed that, in PI cultivar, the majority of proteins involved in heat response and redox have shown either water or heat stress memory. Whereas, in R95 cultivar, the majority of proteins involved in metabolism and photosynthesis have shown either water or heat stress memory, and redox proteins showed heat stress memory in R95 cultivar. Accumulation of transcription factors or proteins facilitates a fast response to repeated stress exposure (Bruce et al., 2007).

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*iii). Effect of heat stress showed high abundance of the heat responsive proteins in PI-cultivar:* The majority of heat response proteins-heat shock protein 70, 22 kDa heat shock protein, 17.7 kDa class 1 small heat shock protein, and 17.6 kDa class 1 heat shock protein, were consistently high in abundance to heat stress in PI cultivar, but were low in R-95 cultivar. HSP70 is one of the most important proteins in the response to heat stress, and studies show that this protein is directly linked to the thermotolerance of the plant (Ahsan et. Al, 2010). HSPs and small HSP make up molecular chaperones and involved in protein folding, prevention of protein aggregation, translocation of proteins across membranes, targeting proteins towards degradation, and regulation of translation initiation, thus the renaturation of stress-damaged proteins protecting cells against the effects of stress (Sarkar, 2013). Some HSPs are also involved in transcriptional activation of additional small HSP promoters (Huang et. al, 2012). Plants up-regulate HSPs, in particular HSP-70, are more tolerant to heat stress (Ray et al, 2016).

*iv) Effect of water stress showed high abundance of proteins involved in metabolism in* R95-1705 *cultivar:* Out of the total 12 proteins involved in the metabolic processes, four proteins were relatively more abundant with 5 proteins maintaining their abundance in R95, only two proteins were more abundant in PI, and two maintained their abundance.

Alanine aminotransferase 2 was the only protein that was more abundant in response to WS in both cultivars. The protein is found in peroxisomes and involved in the degradation of amino acids in plant cells (Hildebrandt et al. 2015). The abundance of these enzymes indicates that the amino acid metabolism and the synthesis of other metabolites derived from amino acids are well maintained under drought stress (Wang et al. 2016). Acid phosphatase was over expressed to WS in R95 and remained unchanged in PI cultivar. The role of acid phosphatase is important in maintaining metabolic homeostasis during drought stress (Kadam et al., 2012).

Serine hydroxymethyltransferase 5, a glycolytic protein involved in photorespiration was not expressed in control plant however was induced to combination of WS+HS stresses in R95 cultivar. These proteins are directly involved in initiation and elongation of the newly growing peptide chains, indicating seriously reduced protein synthetic capacity under drought. Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) is involved in the photorespiratory pathway of oxygenic photosynthetic organisms (Waditee-Sirisattha et al, 2017). SHMT, a pyridoxal phosphate-dependent enzyme, plays a pivotal role in cellular one-carbon pathways by catalyzing the inter-conversion of L-serine to glycine and tetrahydrofolate to 5,10-methylenetetrahydrofolate for synthesis of nucleic acids, and proteins (Mishra et al, 2019).

Translation elongation factor Tu was overexpressed to WS in R95 cultivar. Translation elongation factor Tu is a GTPase that is responsible for delivering amino-acylated tRNAs to the ribosome during translation (Hughes 2017). Nucleoside diphosphate kinase (NDPK) is more abundant in R95 cultivar, and low in PI in response to WS. NDPK is found in the matrix and inner membrane of mitochondria, which regulates the cellular physiology, is known to interact with heat shock proteins (Kosová et al., 2015).

*v) Combined water and heat stress significantly altered metabolism, redox and photosynthesis related proteins in both cultivars:* Eleven proteins (#1, 3, 8, 9, 10, 12, 18, 25, 27, 28, and 36) were more abundant in PI cultivar, with the majority involved in heat response and photosynthesis. Among these, five proteins were in low abundance to WS when applied independently. Twelve proteins (#3, 5, 7, 8, 9, 12, 14, 15, 22, 23, 24, and 31) were more abundant to combined stress in R 95 cultivar. The majority were involved in metabolism, out of which, three proteins were low in abundance to WS and two proteins to HS. Soybean cultivar PI 471938, which exhibits a slow-wilting phenotype under water-deficit conditions, has proven to be a good genetic resource in developing drought resistant progeny (Sadok et al., 2012).

*vi). Protein-protein interactions:* Our studies showed that, more proteins were expressed in higher abundance to combined stresses in PI cultivar and having more promotive interactions with other proteins, and in particular the proteins associated with metabolism, and photosynthesis, leads to higher performance of the plant to the multiple stresses. Protein MED37C (P26413), identified in our studies was also observed to have the good potential interacting partners in *Arabidopsis thaliana*. Protein MED37C (P26413) showed two promotive interactors in both cultivars. In the R95 cultivar, it has more inhibitory interactors (five), suggesting that the effect of the protein interaction is more significant in PI than in R95 cultivar in response to combined stresses. Therefore, we contemplate that the protein might also have potential interacting partners in soybean. This candidate protein would be of potential interest as it is a probable mediator of RNA polymerase transcription II associated with heat shock proteins (Huang and Xu, 2008).

*vii). Co-relation of mRNA expression for selected stress responsive proteins:* The protein abundance levels as a result of differential expression of mRNA transcript levels can often be correlated with their protein expression. Although most of the mRNA expression levels in this study exhibited expression trend that were matching with corresponding protein abundance levels, there is a mixed response in the expression and accumulation pattern of some mRNA and the corresponding protein abundance profiles. In PI cultivar, expression of serine and heat shock protein at both transcript level and protein abundance were corresponding with protein abundance and were increased to all stress treatments. Whereas, the expressions of peroxidase, chalcone flavone isomerase, ascorbate perodxidase, catalase, calireticulin, peroxiredoxin, and superoxide dismutase transcripts were in contrast to their protein abundance. There is conflict as the protein abundance is not correlated with the expression of corresponding transcript levels. In cultivar R 95, the expression levels of transcript follow their relative abundance in proteins. Broadly, catalase, serine, chalcone flavone isomerase were up regulated at transcript level and showed relatively high abundance of proteins in response to the stress treatments, while calireticulin, peroxidase, peroxiredoxin, ascorbate peroxidase, superoxide dismutase, and heat shock protein were low at both transcript and protein levels. Reports indicate a poor correlation between mRNA and protein abundances in the cell and depends on various biological and technical factors (Wu et al., 2008). The correlation may not be as similar because the mRNA transcription is relatively lower than protein translation (Kosti et al., 2016). Protein levels are more conserved than mRNA levels, and their turnover is probably influencing the correlation between mRNA and protein abundances to a greater degree (Doherty et al., 2009).

*viii). Elevated activities in antioxidant enzymes in PI-373819 cultivar enhances the tolerance to ROS production and builds homeostasis:* Antioxidant enzyme activities displayed significantly higher levels in PI cultivar to all stress treatments, despite of their activities low under control conditions when compared with those of R95 cultivar. SOD is considered first line defense against toxic effects of elevated ROS and the increase in SOD in PI cultivar to all three stresses would play major role in ROS scavenging in plants and is considered as the first line of defense against the toxic effects of elevated ROS levels (Gill and Tuteja 2010). SOD catalyzes the dismutation of superoxide radicals to $H_2O_2$ and $O_2$. The increase of SOD activity might be the reason for enhanced $O_2$ generation, as a result of electron leakage from the electron transport chains to molecular oxygen (Yıldıztugay et al., 2011). Water stress or combined WS+HS reportedly induce oxidative stress in plants (Zandalinas et al., 2017). POD is the primary enzyme and the increase in its activity detoxifies $H_2O_2$ in chloroplast and cytosol during oxidative stress (Etemadi et al., 2015). Both POD and CAT constitute a main $H_2O_2$ scavenging system during oxidative stress induced by WS or HS or the both (Akram et al, 2017). Similar observations of higher POD and CAT activities were reported in tolerant genotypes implicating their role in developing resistance to one or more stresses, when compared to the decrease in these enzyme levels in susceptible genotypes (Ara et al. 2013).

APX activity and GR was elevated in PI cultivar to all stresses, while their levels GR was not detected in R 95 cultivar when treated with combined stresses. Overexpression of APX seems to play a key role in regulating of $H_2O_2$ levels in plant cells by preventing $H_2O_2$ from reaching the nuclei from cytosol, preventing lipid peroxidation and protein oxidation and thereby making the cultivar more tolerant (Apel and Hirt, 2004). Increased APX indicate that the PI cultivar has the ability to restore the oxidation levels and sustain the plant during the stress. GR keeps GSH/GSSG ratio favorable for ascorbic acid reduction. The GR activity in PI cultivar preserved GSH/GSSH ratio due to lower incidence of oxidative damage. In contrast, the abence of GR activity in R95 cultivar in response to combined stress suggest impairment of GSH recycling due to enhanced ROS accumulation (Foyer and Noctor, 2011).

**Conclusions:**

This study provides insights into proteome and enzyme responses to multiple stresses occurring simultaneously in the field conditions. Results showed concurrent stresses alter physiological, proteome and enzymes indifferent to individual stress. Cultivar PI-371938 maintained total biomass to all stresses, compared to R95-1705 cultivar. Degree of genetic diversity was observed between two cultivars in their protein abundance when subjected to various types of stresses. Several proteins involved in metabolism, response to heat and photosynthesis have shown significant cross-tolerance mechanism. Cross-tolerance was evident among heat responsive proteins, photosynthesis, metabolism and redox proteins that were high in abundance to heat stress as well as to the combined heat and water stress in the PI-371938 cultivar suggesting the cultivar as relatively heat tolerant. Cross-tolerance was observed in the proteins involved in metabolism, and photosynthesis in the R95-1705 cultivar in response to water stress or in combination of water and heat stress. Both sets of proteins were adversely affected when treated with heat stress alone. However, heat stress alone, enhanced redox related proteins in R95 cultivar.

Elevated activities in antioxidant enzymes, such as increased APX indicate that the PI-371938 cultivar has the ability to restore the oxidation levels and sustain the plant during the stress. Proteins were elevated in high abundance to combined stress in PI-371938 demonstrated more promotive interactions associated with metabolism, photosynthesis leading to sustained resistance the both types of stress. Protein MED37C, a probable mediator of RNA polymerase transcription II yielded potential protein interactors partners in *Arabidopsis* and our studies documents the significant impact of the protein in PI cultivar. Levels of protein expression and transcripts correlate with the regulation at transcription and post transcription levels. Furthermore, the milder stress on small scale can mitigate the detrimental effect of extreme conditions.

**Acknowledgements:**

RK, NM, VP acknowledges the financial support from National Science Foundation Grant number 1156900 to carry out analysis of this research. Ms. Tiffany Boyunt reviewed and assisted in preparing graphs. Ms. Catlyn Eliason and Ms. Sara Bergin read and edited the MS.

**Author contributions**

KR performed the laboratory experiments and wrote the manuscript draft and executed the research, NM carried out analysis of the 2D gel data; SS revised the manuscript, collected the literature for data discussion; SL performed the field experiments and collected the plant material;

PS conducted interactome data analysis and edited the manuscript; MK conducted Mapman bin analysis and edited the manuscript; KS carried out mathematical prediction of protein interactions and edited the manuscript; MB prepared figures and edited the manuscript; SS and VI prepared tables, and edited the manuscript; KRR conceived the project, executed, and supervised this research.

**Conflict of interest:**

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

**Declarations:**

The corresponding authors certify that all authors have seen and approved the final version of the manuscript being submitted and warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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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 | TBM |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PI 373819 | 28/20 | WW | $0.0855_{bc}$ | $83.1_{a}$ | $37.4_{c}$ | $28.82_{a}$ | $1.399_{b}$ | $0.557_{a}$ | $353_{ab}$ | $16.11_{c}$ | $14.48_{a}$ |
| | 28/20 | WS | $0.0045_{d}$ | $71.1_{cd}$ | $39.6_{bc}$ | $1.95_{de}$ | $0.055_{c}$ | $0.435_{b}$ | $323_{abc}$ | $1.66_{d}$ | $10.28_{abc}$ |
| | 38/30 | WW | $0.0899_{ab}$ | $76.5_{bc}$ | $39.5_{bc}$ | $27.72_{ab}$ | $1.866_{a}$ | $0.543_{a}$ | $362_{a}$ | $27.51_{b}$ | $13.47_{ab}$ |
| | 38/30 | WS | $0.0013_{d}$ | $64.2_{e}$ | $39.6_{bc}$ | $0.67_{e}$ | $0.022_{c}$ | $0.325_{c}$ | $319_{b}$ | $1.28_{d}$ | $9.00_{bc}$ |
| R95 – 1705 | 28/20 | WW | $0.0962_{a}$ | $80.6_{ab}$ | $40.0_{abc}$ | $24.79_{c}$ | $1.308_{b}$ | $0.544_{a}$ | $350_{ab}$ | $16.81_{c}$ | $13.33_{ab}$ |
| | 28/20 | WS | $0.0033_{d}$ | $70.1_{d}$ | $39.5_{bc}$ | $3.28_{d}$ | $0.054_{c}$ | $0.396_{b}$ | $287_{c}$ | $1.72_{d}$ | $7.59_{c}$ |
| | 38/30 | WW | $0.0829_{c}$ | $79.5_{ab}$ | $43.8_{a}$ | $25.86_{bc}$ | $1.810_{a}$ | $0.551_{a}$ | $360_{a}$ | $32.08_{a}$ | $13.86_{a}$ |
| | 38/30 | WS | $0.0013_{d}$ | $63.7_{e}$ | $41.5_{ab}$ | $2.60_{de}$ | $0.015_{c}$ | $0.331_{c}$ | $193_{d}$ | $0.84_{d}$ | $6.79_{c}$ |
| | *ANOVA* | *ANOVA* | | | | | | | | | |
| | | C | ns | ns | * | ns | ns | ns | *** | ns | ns |
| | | T | * | ** | ns | ns | * | *** | * | *** | ns |
| | | IRR | *** | *** | ns | *** | *** | *** | *** | *** | *** |
| | | C □ T | ** | ns | ns | ns | ns | ns | * | ns | ns |
| | | C □ IRR | ns | ns | ns | ** | ns | ns | *** | * | ns |
| | | T □ IRR | ns | ns | ns | ns | ** | *** | ** | *** | ns |
| | | C □ T □ IRR | ** | ns | 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 $\alpha = 0.05$

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| 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 |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***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*** | | | | | | | | |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 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 |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 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***

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 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 |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 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
bProtein identification number as in Uniprot/NCBI database
c Protein identification number as in Soybean phytozyme database
dProtein molecular weight
epI value
f Theoretical value
g Experimental Value
hIdentified peptide coverage
* Protein resolved in multiple spots on 2DE