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Proteomic Analysis in Wheat to Study the Effect of Heat Stress on Flag Leaf

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A B S T R A C T

The analysis of abiotic stress responsiveness in plants is a very crucial way to identify the genes conferring stress tolerance and their use in crop improvement programme. Heat stress is one of the stresses which highly affect the growth, quality character as well as total yield of the crop. In this study, a proteome analysis by 2D electrophoresis was used to identify the characteristic change in two wheat genotypes *viz*, GW 451 (heat tolerant) and WH 147 (heat susceptible) from flag leaf. Control and treated leaf samples showed the expression of differentially expressed proteins at booting stage in both genotypes of wheat. After the completion of 2DE, expressed protein were analysed as spot formation on gel. The comparison of two different gel as control and treated for each genotypes gave the information regarding molecular weight, pH, volume of that protein etc. A total of 568 spots were detected in both genotypes among which higher molecular weight spots, 102 kDa and 107 kDa were reported in treated leaf samples of GW 451, while WH 147 contains more numbers of less molecular weight proteins. In this study, various proteins were reported with highly versatile amount of molecular weight. Result of proteomics indicated the tolerance level against heat stress. Comparative proteomic study of plants with and without heat stress allows us to obtain various information regarding how plant adopted defence mechanism against heat stress condition.

Introduction

Every living organism, especially plants, has the ability to cope up with any stressed condition. Immobility of the plants allowed them to develop various proteins which give them the ability to escape from various stressed conditions.

These sets of proteins protect the cells from damage. The quality and yield of potential various cereals are highly affected by heat stress in many countries (Treglia *et al.*, 1999). Wheat is one of the most important crops, after rice and maize, in the world.

Common bread wheat is used worldwide due to its beneficial globular protein, Gluten and starch. Thus, to understand the effect of high temperature on wheat, it became important to improve the quality and yield of wheat for various warm as well as temperate countries.
In plants, the heat shock responses are result of altered gene expression and protein

translation from ubiquitous phenomenon. There are various types of heat shock proteins as high molecular weight HSPs (80-100 kDa), intermediate molecular weight HSPs (68-73 kDa) and small molecular weight HSPs (15-20 kDa) (Vieling, 1991; Sun *et al.,* 2002).

Apart from being synthesized as heat shock protein, HSPs are also accumulated in plants in response to a large number of other stress factors such as arsenite, ethanol, heavy metals, water stress, light, hormones, abscisic acid, wounding, excess NaCl, chilling, and anoxic conditions (Prasad and Rengel, 1998; Anderson *et al.,* 1994; Lee *et al.,* 1996; Sabehat *et al.,* 1998).

Proteomics is a very elegant approach for understanding of cellular processes. This tool provides more fundamental insights into organism development and homeostatic control than provided by the genome sequence (Shihua *et al.,* 2003).

Proteomic responses to heat stress have been investigated in a number of plants, rice (*Oryza sativa* L.) (Lin *et al.,* 2005; Lee *et al.,* 2007); wheat (*Triticum aestivum* L.) (Skylasa *et al.,* 2002; Majoul, *et al.,* 2003; Laino *et al.,* 2010); barley (*Hordeum vulgare* L.) (Sule *et al.,* 2004); and maize (*Zea mays*) (Hu *et al.,* 2010; Liu *et al.,* 2013) and many stress-related proteins have been identified.

In a differential analysis set-up, protein profiling method provides powerful insights into the stress responsiveness of genes (Zivy and De-Vienne, 2000).

2D electrophoresis has been widely used for the separation of proteins and examines the expression of those proteins. The present study provide the protein based changes under heat stress condition in susceptible and resistance genotype of wheat which plays an importance role for food security.

Materials and Methods

Sample collection and preparation

The experiment was carried out at Department of Biotechnology, Collage of Agriculture, Junagadh Agricultural University, Junagadh during 2016-17. Two wheat genotypes, GW 451 (heat tolerant) and WH 147 (heat susceptible) were used for experiment. Seeds of both genotypes were grown up to boot leaf stage (around 52 days after sowing). Flag leaves from both genotypes were collected as control samples and other set of plants were treated at 40ºC for one hour and treated leaves were collected as treated samples.

Protein extraction

Collected leaf samples were stored in -20ºC refrigerator for further analysis. Leaves were first ground in chilled mortar containing liquid nitrogen until a fine powder was obtained and then the proteins were extracted following the procedure described as sample was precipitated in trichloroacetic acid (TCA)-acetone (Weng *et al.,* 1992). Precipitated proteins were centrifuged at 19,000 g for 1 h at 4°C and were washed thrice with ice-cold acetone containing 0.07% (v/v) β-mercaptoethanol. Extracted proteins were dissolved in 800 µl of strip rehydration buffer containing 7M Urea, 2M thiourea, 2%w/v CHAPS, 2%v/v Triton X-100, 1.2% v/v Destreak reagent (GE Healthcare), and 0.5% v/v IPG buffer pH 3-10. The suspension was incubated at 4°C for 1.5 h and then centrifuged at 19,000 g for 1h at 4°C to remove any insoluble material. The supernatant containing soluble proteins was quantified by a 2-D Quant Kit (GE Healthcare Life Sciences) using bovine serum albumin (BSA; 1mg/ml) as a standard. The quantified protein samples were stored in aliquots at—80°C until analysis by two-dimensional gel electrophoresis (2-DE).

Two-dimensional electrophoresis (2-DE), gel staining and image analysis

The first dimension of 2-DE was performed on immobilized pH gradient (IPG) strips 3-10. Total volume of sample 200 µl was pipetted out along edge of the rehydration tray and evenly distributed except for about 1 cm at each end. For the first dimension electrophoresis, 350 µl IEF rehydration buffer (7M urea, 2M thiourea, 4% (g/ml) CHAPS, 65 mM DTT, 0.2% (g/ml) bio-lyte, and 0.01% (g/ml) bromophenol blue) containing 600 µg (leaf) protein was loaded onto the IPG strips and 2 to 3 ml of mineral oil was overlaid on each strip to prevent the evaporation during rehydration process. Appropriate 6 step protocol was programmed for the Ettan IEF cell.

Default cell temperature of 20°C with maximum current of 75 ìA per strip was used. The running conditions were as followed: 200 V for 1 hr, 500 V for 7 hrs, 1000 V for 1 hr followed by gradient 8000 V for 8 hrs, 8000 for 5 hrs and finally 500 V for 4 hrs. Focused strips were incubated in 10 ml of equilibration buffer-I containing Urea (6M), 2% SDS, Tris buffer (75 mM, pH- 8.8), 87% glycerol and DTT (100 mg per 10 ml buffer) for 15 minutes. After that equilibration buffer-I was drained off and again 10 ml of equilibration buffer-II containing Urea (6M), 2% SDS, Tris buffer (75 mM, pH- 8.8), 87% glycerol and iodoacetamide (250 mg per 10 ml buffer), was added to the strip and kept on shaker for 15 minutes with continuous shaking. The second dimension of 2-DE was performed on a 12% SDS-PAGE running gel. Separation of proteins in 2-D gel electrophoresis was similar as that of the SDS-PAGE. After completion of electrophoresis, for the visualization of protein spots, gel was stained by CBB staining according to Neuhoff *et al.,* (1988). Gels were stained for 16h and destained for 1h with distilled water before image acquisition. Gel was then scanned through Typhoon FLA 7000 scanner and photographs were used for further analysis through Image master 2-D platinum software powered by Melanie.

Results and Discussion

The estimation of protein by Bradford's method showed higher numbers of proteins in control leaf samples as compared to treated leaf samples in both genotypes. While in case of genotypes, GW 451 showed more protein content as compared to WH 147 (Table 1). Kumar *et al.,* (2013) used SDS-PAGE for determination of protein content from leaf samples of wheat under heat stress and observed the higher expression of proteins in controlled samples while lower expression in heat treated leaf samples.

There are four different comparison was done using 2-DE which showed altered protein expression in control and treated leaf samples of wheat flag leaves. Figure 1(I-IV) showed the detected protein spots examined after 2D electrophoresis. Variation showed the changes in protein levels during stressed conditions.

In case of control and treated leaf samples of WH 147, more than 200 spots were identified, among which 30 spots were common in both gels. Out of these 30 spots, 17 were down regulated and 13 were up regulated in treated leaf samples. In comparison of control and treated leaf samples of GW 451, 242 spots were detected, out of which 45 were common in both samples, 22 up regulated and 22 down regulated. Only one protein was reported in both genotypes which neither up regulated nor down regulated, the value remain same in both gels.

There were 257 spots were identified in comparison of control leaf samples of GW 451 and WH 147, in which 31 spots were common in both genotypes.

Table.1 Estimation of Protein content by Bradford's method

Genotype	Control	Treated	Mean	t-Test (0.05)
GW 451	33.36 ± 0.02	27.56 ± 0.02	30.46 ± 0.09	6.34*
WH 147	24.13 ± 0.02	20.63 ± 0.03	22.38 ± 0.09	
Mean	28.60	24.10	-	-
t- test (0.05)	3.68*		-	-

Table.2 Comparative analysis of level of expression between treated and control leaf of WH 147

Sr. No.	Match ID	Coefficient of Variation (%)	WH 147 (Control leaf)			WH 147 (Treated leaf)		
			pI	MW (kDa)	% vol	pI	MW (kDa)	% vol
1	152	48.5	8.97	14	0.770	10.11	60	0.267
2	154	51.6	7.78	16	0.125	9.21	44	0.394
3	156	29.9	8.47	18	0.404	9.49	60	0.750
4	157	52.7	8.19	23	0.295	8.97	61	0.954
5	161	50.2	5.98	50	1.013	6.39	38	0.337
6	162	38.0	6.63	58	1.427	6.58	60	0.642
7	163	48.0	8.71	60	1.028	7.69	97	0.362
8	169	18.4	4.34	77	0.379	4.59	22	0.550
9	172	40.6	5.67	95	1.268	5.10	52	0.537
10	176	82.0	4.35	97	0.245	3.85	31	2.480

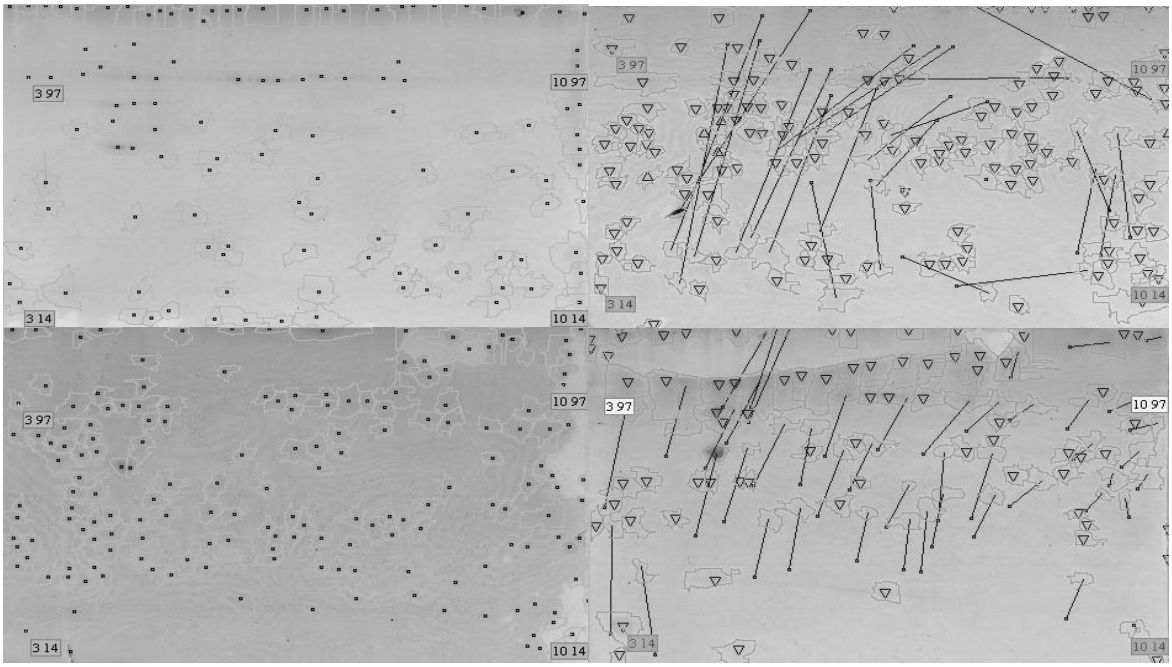
Table.3 Comparative analysis of level of expression between treated and control leaf of GW 451

Sr. No.	Match ID	Coefficient of Variation (%)	GW 451 (Control)			GW 451 (Treated)		
			pI	MW (kDa)	%Vol	pI	MW (kDa)	%Vol
1	48	18.7	4.49	36	0.191	4.83	66	0.130
2	50	31.2	7.74	37	0.309	8.07	63	0.589
3	56	51.5	9.93	47	0.280	10.02	53	0.876
4	68	71.1	4.53	69	0.831	4.86	102	4.913
5	69	39.4	7.67	77	0.207	8.16	97	0.477
6	72	35.3	4.82	83	0.400	5.38	97	0.191
7	73	01.4	8.25	84	0.470	8.61	95	0.484
8	74	38.8	9.56	91	1.079	9.94	97	2.445
9	75	52.6	4.85	94	0.575	5.09	97	0.178
10	76	20.8	4.48	94	0.847	4.93	97	0.555

Table.4 Numbers of spots identified in 2DE in control and treated leaf sample in both genotypes

	Control Leaves		Treated Leaves		Total No. of Spots	
	GW 451	**WH 147**	**GW 451**	**WH 147**	**GW 451**	**WH 147**
PI (2-4)	41	17	21	28	62	45
MW (KDa)	14-97	14-97	14-102	14-99		
PI (4-6)	35	41	30	45	65	86
MW (KDa)	18-97	12-97	14-107	14-97		
PI (6-8)	41	27	27	40	68	67
MW (KDa)	17-97	12-100	18-97	13-97		
PI (8-10)	53	33	38	51	91	84
MW (KDa)	12- 97	14-97	14-97	14-97		
Total Spots	170	118	116	164	286	282
Total	**288**		**280**		**568**	

Fig.1 Spots detected on gel photograph of A) control leaf sample of heat susceptible genotype (WH 147); B) treated leaf sample of heat susceptible genotype (WH 147); C) control leaf sample of heat tolerant genotype (GW 451); D) treated leaf sample of heat tolerant genotype (GW 451)



On the other hand, there were only 20 spots, out of 257 spots, were common in treated leaf samples from both genotypes in which 13 were up-regulated in GW 451, while rest were down regulated. Susceptible genotype WH 147 showed variation in protein expression under control (Fig. 1-I) as well as heat stress condition (Fig. 1-II). Match ID 152, 154, 156, 157 were correspondence with WH 147 genotype during heat stress conditions as the molecular weight was gradually increase under heat stress condition, while in some case of proteins the molecular weights were gradually decreased, for example Match ID

spots as 161, 162, 169, 172, 176. In tolerant genotype GW 451, out of 45 matched spots, Match ID No. 48, 50, 56, 68 indicated boosting of proteins in treated leaf samples as compared to control leaf samples. The comparative analysis of control leaf samples of both genotypes showed various spots out of which 31 spots were matched. Match ID 130, 132, 135, 140 indicated the increased and decreased amount of protein molecular weight in GW 451 as compared to WH 147. While treated leaf samples of both genotypes showed 20 matched spots. Treated spot Match ID 96, 92 and 97 showed highest molecular weight 102 kDa, 105 kDa and 107 kDa, respectively.

By spot-to-spot comparisons, we identified a total of 568 protein spots in both genotypes as 286 spots in GW 451 and 282 spots in WH 147. Out of which 170 spots were noted in control leaf samples of GW 451 and 118 in control leaf samples of WH 147. On the other hand, for treated leaf samples only 116 spots were reported in GW 451, while 164 spots were reported in WH 147. Differential expression of proteins were determined by up and down regulation of spots as 35 proteins were up regulated in treated leaf samples and 39 were down regulated in control leaf samples. Highest numbers of spots were reported between pH 8 and 10 in GW 451, while lowest numbers of spots were reported between pH 2 and pH 4 in WH 147 (Table 2). In this study, we identified different spots which were indicative of various numbers of small heat shock proteins, as well as intermediate and large heat shock proteins. Treated leaf samples from both genotypes showed more numbers of large heat shock proteins as compared to control leaf samples.

There had been various research work on wheat protein for identification of their involvement into various pathways by their expression analysis. Liu *et al.,* (2014) reported variable numbers of spots with up and down regulation of leaf samples of wild wheat cultivars in which few spots were up regulated after 12h of treatment but those proteins were down regulated after 48h of treatment. Contradictory scenario was also reported for few numbers of proteins. They also identified various functional proteins to escape the stress with versatile molecular weight. More numbers of small heat shock proteins were noted in their study. Cheng *et al.,* (2015) studied expression level of leaf proteins in wheat and showed 29 spots common in both genotypes out of which 11 were up regulated and the rest were down regulated in leaf samples. They also reported 77 unique proteins in CS and NC47 cultivars (Table 3).

Kumar *et al.,* (2015) also noted 216 protein spots out of which 111 spots were present in control samples, while 105 spots were reported in heat stress treated samples of wheat. They also reported 44 unique spots in control leaf samples and 38 unique spots in heat stressed samples along with 12 up regulated and 7 down regulate proteins related to heat stressed. Irar *et al.,* (2010) also reported heat shock proteins in two genotypes of wheat which ranged between small HSPs to intermediate HSPs, while, Han *et al.,* (2009) reported protein spots which ranged from 14 kDa to 90 kDa in rice (Table 4).

It is concluded that GW 451 showed more rapid growth than WH 147. The estimated protein isolation was higher in control leaf samples as compared to treated leaf sample in both genotypes. While in comparison of both genotypes, proteins reported in heat tolerant genotype was higher than heat susceptible genotype. Using 2D electrophoresis various proteins were identified as 288 spots in GW 451 and 280 spots in WH 147 which includes good number of heat shock proteins. In GW 451, the highest number of proteins was 91

protein spots with pH ranged from 8 to 10. While in WH 147, the highest number of protein spots was 86 with pH range from 4 to 6. This study of 2-DE need to identify various proteins which could have crucial role in heat stress response, the data can be used for further studies which specifically focus on tolerance mechanism of wheat under heat stress condition.

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