Australian Journal of Crop Science AJCS
AJCS 8(6):973-986 (2014) ISSN:1835-2707

Proteome analysis of wheat (*Triticum aestivum*) for the identification of differentially expressed heat-responsive proteins

Ranjeet Ranjan Kumar[1,*], Gyanendra Pratap Singh[2], Suneha Goswami[1], Himanshu Pathak[3] and Raj Deo Rai[1]

[1]Division of Biochemistry, Indian Agricultural Research Institute, New Delhi, India Pin 110012
[2]Division of Genetics, Indian Agricultural Research Institute, New Delhi, India Pin 110012
[3]CESCRA, Indian Agricultural Research Institute, New Delhi, India Pin 110012

***Correspondence: ranjeetranjaniari@gmail.com**

Abstract

Heat stress is one of the major problems in wheat growth and yield. It causes denaturation and aggregation of key enzymes involved in different pathways and affects the quality of the grains. The defence mechanism associated with thermotolerance in wheat is not known and very few stress associated proteins has been characterised. We performed an extensive identification of heat-responsive proteins in control (22°C) and heat shock (HS) treated (42°C, 2 h) leaf samples of HD2985 cultivar of wheat at milky-ripe stage using 2-DE coupled with MALDITOF/MS, nLC/MS and MS2. Orbitrap was used because of its highest rate of true protein identification and speedy processing of samples. We identified 47 and 38 unique protein spots with 19 differentially expressed in control and heat shock (HS) treated samples. Similarly, using Orbitrap we could able to identify 97 peptides, 60 proteins (control) and 262 peptides, 135 proteins (HS) using protein database of *Arabidopsis thaliana* and 317 peptides, 164 proteins (control) and 592 peptides, 263 proteins (HS) using protein database of *Oryza sativa* (NCBI RefSeq). Differentially expressed proteins (DEPs) were identified as HSP70, Oxygen evolving enhancer protein, Calmodulin, Hypothetical protein, stress predicted protein, Rubisco, Rubisco activase etc. Real-Time expression profiling showed abundance of HSPs (*HSP26*, *HSP70*) and signalling molecule (*CDPK*) in response to HS, whereas down-regulation was observed in case of starch biosynthesis pathway associated gene. Novel heat stress associated proteins (HSAPs) can be used as marker in back-cross breeding program for the development of introgressed lines with desired traits for thermo-tolerance. Novel proteins and their respective genes can be manipulated for the development of heat tolerant wheat.

Keywords: DEPs; Heat stress; Heat-responsive proteins; MALDI-TOF; nLC-MS/MS; Orbitrap; qRT-PCR; Transcript; Wheat.
Abbreviations: CDPK-calcium dependent protein kinases; DEPs-differentially expressed proteins; HS- heat stress; LC-MS/MS-liquid chromatography mass spectrometry; nLC/MS2-nano liquid chromatography mass spectroscopy; qRT-PCR-quantitative real-time PCR; SAP-stress associated protein.

Introduction

Elevation in temperature above ambient reduces the growth and yield of agriculturally important crops especially cereals. Wheat is highly sensitive to heat stress and even slight variations in temperature during critical stages like pollination and milky-ripe reduces the quality and quantity of wheat grains. Increase in temperature of 1°C reduces the yield of wheat by 4% (Kumar et al., 2013a). Heat stress causes pollen sterility, drying of stigmatic fluid, incomplete fertilization, pseudo-seed setting, defragmentation of starch granule, empty pockets synthesis in endosperm and shrivelled grains (Kumar et al., 2013b). On quality aspect, the grains harvested from plants exposed to heat stress have been reported to have low nutrient quality as well as dough making properties. Some of the defence mechanism which has been characterised in cereals is oxidative burst of reactive oxygen species (ROS), over-expression of signalling molecules and kinases, enhance in the expression of heat-responsive transcription factors and stress associated genes etc. (Sairam et al., 2000; Kumar et al., 2012). These stress associated genes (SAGs) and proteins (SAPs) has been fully characterized in other crops like rice (*Oryza sativa*), maize (*Zea mays*), sorghum (*Sorghum bicolor*) etc., but in wheat, the mechanism has not been deciphered because of few reasons like the genome is partially sequenced, hexaploid and complex nature of genome, availability of limited information's regarding stress associated proteins etc. The first draft of wheat genome has been recently sequenced and it has open a new area of research to decipher the defence pathways associated with abiotic stress tolerance in wheat (Ling et al., 2013). Limited information's are available on the stress associated proteins (SAPs) identified in wheat (*T. aestivum*). Now with the advent of second generation proteomic tools (2-DE and MALDI-TOF-MS, nLC/MS2), new research work has been initiated for identification of novel stress-responsive proteins in different crops. Differential protein profiling and MALDI-TOF mass spectrometry identification of protein has paved the way for exploring the different pathways, but then due to lack of high

resolution, the number of identified protein match-set is very low. New proteomic tools like label-free nano LC-MS and MS2 (nLC/MS2) allows to identify novel proteins at very high resolution (Vensel et al., 2002). Mass spectrometry (MS) has ample of choices to use different analysers like quadrupole, magnetic sector, ion trap, time-of-flight (TOF), or Fourier transform (FT) generic types. The orbitrap analyser has been introduced in the year 1999, and has been accepted in mainstream MS as an accurate and compact mass detector. The technique is used in the form of a hybrid instrument (LTQ Orbitrap) featuring a linear ion trap front-end. Some of the characteristic of this instrument is image current detection from Fourier Transform Ion Cyclotron Resonance (FTICR), the use of ion trapping in precisely defined electrode structures from the radiofrequency ion trap, pulsed injection and the use of electrostatic fields from the TOF analysers (Makarov et al., 2006). Oliveira et al. identified numerous heat-stable Lea proteins in *Arabidopsis thaliana* using 1D SDS-PAGE coupled to LC-ESI-MSMS analysis and a gel-free protocol associated with LC-MALDI-MSMS (Oliveira et al., 2007). Two-dimensional electrophoretic analysis revealed about 150 polypeptide spots in the pH range of 3.0 to 10.0 in salt treated tobacco leaf protein extracts (Dani et al., 2005). iTRAQ together with 2D-LC and ESI-MS/MS reproducibly identified 122 proteins with confidence ≥ 95% ($\rho < 0.05$) in *Xerophyta viscosa*. Classification of the nuclear proteins according to Gene Ontology (GO) annotation showed that most proteins were part of cellular processes (77.43%) and had binding activity (85.47%) respectively (Abdalla et al., 2012). One hundred and ninety eight DEPs were located in wheat with at least two-fold differences in abundance on 2-DE maps, of which 144 were identified by MALDI-TOF-TOF MS (Guo et al., 2012). These proteins were involved primarily in carbon metabolism (31.9%), detoxification and defence (12.5%), chaperones (5.6%) and signal transduction (4.9%). Liu et al. identified 22 major differentially expressed proteins in response to heat and drought stress in maize roots using mass spectrometry (Liu et al., 2013). Mass spectrometry (MS) and immunoblotting has been used extensively to assess for the presence of specific protein fragments (Boye et al., 2013). Haddad et al. identified thirty-eight HS responsive albumin and globulin proteins, including several enzymes involved in carbohydrate, redox, and lipid metabolisms in wheat using 2-DE followed by MALDI-TOF/MS (Haddad et al., 2013). In present investigation, we have used these novel technologies to identify heat-responsive proteins in HD2985 (thermotolerant) cultivar of wheat grown under heat stress. 2-DE coupled with MALDI-TOF/MS, nano LC/MS and MS2 were used for the polypeptide identification. The identified peptides and proteins were further characterised for their role in thermotolerance mechanism in wheat. Validation was also carried out using quantitative real time PCR (qRT-PCR) in response to differential heat shock (HS) treatment.

Results and Discussion

Wide-diversity has been observed in wheat with reference to thermotolerance capacity and different physiological, biochemical and molecular parameters has been used for screening large germplasm for heat stress tolerance (Sairam et al., 2000). The mechanism of thermotolerance in wheat has not been fully deciphered and many of the key components involved in the defense mechanism are unknown.

Differential expression profiling under heat stress using 2-DE

The samples collected from control (22°C) and heat shock treated (42°C, 2 h) HD2985 cultivar of wheat were subjected to 2-DE for profiling the differentially expressed proteins in response to heat stress. A marked variation in the protein spots were observed on the gel in both the control and HS treated samples (Fig. 1a and 1b). Appearance of many new protein spots were observed in HS treated sample compared to control. An increase in the expression of existing proteins was also observed in HS treated sample which is in conformity with the observations made earlier (Kumar et al., 2013a). Liano *et al*. reported that heat stress alters significantly the durum wheat seed proteome in the range between 1.2- to 2.2-fold (Laino et al., 2010). They also revealed 132 differentially expressed polypeptides, 47 of which were identified by MALDI-TOF and MALDI-TOF-TOF MS and included HSPs, proteins involved in the glycolysis and carbohydrate metabolism, as well as stress-related proteins.

2-DE gel image analysis

The SDS-PAGE gels were stained in silver and their respective images were analyzed on IMP7 software (GE Health Care, Amersham). The gels (control and HS treated) were superimposed and further snap-shot was taken from different areas. The total spots detected are shown in figure 2a and 2b and we could also identify the matched spots which were common in both the gel (Fig. 2c and 2d). A total of 216 protein spots were detected in both the gels. The protein spots observed in control and HS treated gels were 111 and 105. The control and treated gels were matched using IMP7 software. The superimposed gels showed 67 spots to be matched in both the gels. The un-matched spots in each gel were further evaluated and the significantly resolved spots were considered as unique spots in their corresponding gels (Table 1). We could observe 44 and 38 unique spots in control and HS treated gels. Software based analysis showed 12 and 7 spots to be up-regulated and down-regulated (Table 2). Match ID 32, 28, 18, 19, 11, 12 and 64 showed down-regulations compared to ID 17, 43, 46, 45, 0, 38, 7, 39, 25, 3, 60 and 42 which showed up-regulation under heat stress. Match ID 42 showed maximum up-regulation (4.2 fold) and ID 32 showed minimum fold change in expression (0.3). A list depicting unique spots as well as differentially expressed protein spots has been shown in supporting document (Supplementary Table 1). A 2D-gel electrophoresis of rice pollen exposed to heat stress showed 46 protein spots changing in abundance, of which 13 differentially expressed protein spots were analyzed by MS/MALDI-TOF and reported to be cold and heat shock protein in N22 (Jagadish et al., 2010).

MALDI TOF/TOF/MS analysis

Based on the calculated fold change in expression as well as appearance of unique spots in control and heat shock treated gels, 15 spots were selected [Spot ID 16,14,32,35,36,74

Table 1. Summary of protein spots identified in 2-DE gels of control (22°C) and heat shock (42°C, 2 h) treated samples of HD2985 (thermotolerant) cultivar of wheat.

Match-set Name	Protein spots	
	Control	Heat shock treated
Total protein spots	111	105
Unique protein spots	44	38
Spots present in both control and HS treated	67	
Differentially expressed protein spots*	12 (up-regulated) and 7 (down-regulated)	

*Protein spots with fold expression ≥ 1.5 and ≤ 0.5 were considered as up-regulated and down-regulated.

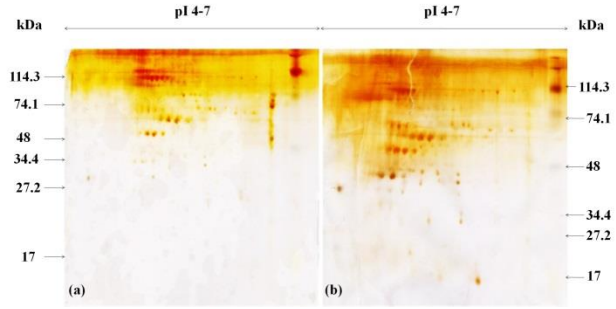

Fig 1. Differential protein profiling of control (22°C) and heat shock (42°C, 2 h) treated samples of HD2985 (thermotolerant) cultivar of wheat by two dimensional electrophoresis (2-DE); (a) 2-DE gel of control sample and (b) gel of heat shock treated sample; the purified proteins were separated on 17cm 4-7 pI IPG strip (Serva, Germany) in the first dimension and 10% SDS PAGE in the second dimension.

(Control) and spot ID 164,120,84,15,20,45,118,66,52 (treated)] for MALDI analysis. These spots were cut, trypsin digested and analyzed by MALDI TOF/TOF (Bruker Daltonics). The raw spectra were smoothed and background removed in Flex analysis software. Thus from the processed spectra the resulting mass list were generated. The m/z value, signal to noise ratios and intensities of each peak for every spectrum were estimated and represented in supporting document [Supplementary Fig. 1(a - p)]. Identification of protein spots were carried out by the database search with MASCOT (Matrix Science) search engine fed from BioTools software (Bruker Daltonics). The identified proteins (Table 3) were additionally searched for sequence similarity protein blast (NCBI) and biological reported functions from UNIPROT. Spot-14 showed maximum identity (61%) with predicted protein of 74 kDa present in sorghum (Supplementary Fig. 1a). Similarly, spot-15 and spot-16 showed maximum homology with ATP synthase alpha subunit (mol. wt. 55 kDa) and hypothetical protein (mol. wt. 18 kDa) reported from wheat and chlorella (Supplementary Fig. 1b & 1c). Li et al. reported approximately 440 differentially expressed protein spots in response to salt stress, heat shock and their combination, and identified 57 proteins using MS (Li et al., 2011). Most of the proteins were functionally involved in disease / defense, photosynthesis, energy production, material transport, and signal transduction. Spot-20 was identified as ATP synthase, whereas spot-32 showed maximum homology with fructose bisphosphate aldolase (mol. wt. 42 kDa) (Supplementary Fig. 1d & 1e). The study of the response of proteins from total endosperm under heat stress identified 36 protein spots to be up-regulated and one spot to be down-regulated (Majoul et al., 2004). Among the heat induced proteins were several HSPs belonging to HSP90, HSP70 and sHSP families, other stress related proteins and enzymes involved in general cellular metabolism. The down-regulated protein was identified as glucose-1-phosphate adenyltransferase, which is known to play a role in starch synthesis (Majoul et al., 2004). Spot-35 and spot-52 were identified as PREDICTED: oxygen-evolving enhancer protein 1, chloroplastic-like isoform 1 reported from *Brachypodium distachyon* (Supplementary Fig. 1f & 1i). Spot-164 showed maximum homology with Ribulose bisphosphate carboxylase small subunit (mol. wt. 13 kDa) (Supplementary Fig. 1p). Most of the other spots were identified as hypothetical or predicted proteins with molecular weight in the range of 16 to 104 kDa and reported from different crops like rice, maize and wheat etc. Rubisco activase was observed to be one of the prominent stress associated protein to be expressed in heat shock treated sample. The identification of 82 of differentially expressed proteins using mass spectrometry revealed a coordinated expression of proteins involved in leaf senescence, oxidative stress defense, signal transduction, metabolisms and photosynthesis which might enable wheat landrace N49 to efficiently remobilized its stem reserves compared to N14 (Bazargani et al., 2011). The list of identified proteins using MALDITOF/MS has been shown in supporting document (Supplementary Table 2).

Identification of differentially expressed peptide and proteins using orbitrap

The HD2985 control (22°C) and HS (42°C, 2 h) treated samples were used for the identification of differentially expressed peptide using second generation proteomic tool- orbitrap. The purified samples were subjected to MS/MS analysis. We could observe 97 and 262 peptides in control and HS treated samples (Fig. 3). Further analysis showed 25, 190 and 72 peptides in control, HS treated and in both the samples. The peptide

Table 2. The matched IDs and relative change in fold expression of respective protein spots in control (22°C) and heat shock (42°C, 2 h) treated gel images of HD2985 cultivar of wheat.

Match ID	% Volume in treated	% Volume in control	Ratio of fold change	Expression
32	17	55	0.309091	Down-regulated
28	15	33	0.454545	Down-regulated
18	16	34	0.470588	Down-regulated
19	12	25	0.48	Down-regulated
11	17	35	0.485714	Down-regulated
12	17	35	0.485714	Down-regulated
64	25	51	0.490196	Down-regulated
17	25	17	1.470588	Up-regulated
43	35	23	1.521739	Up-regulated
46	83	49	1.693878	Up-regulated
45	34	20	1.7	Up-regulated
0	24	14	1.714286	Up-regulated
38	40	23	1.73913	Up-regulated
7	44	25	1.76	Up-regulated
39	38	21	1.809524	Up-regulated
25	53	29	1.827586	Up-regulated
3	66	36	1.833333	Up-regulated
60	56	28	2	Up-regulated
42	101	24	4.208333	Up-regulated

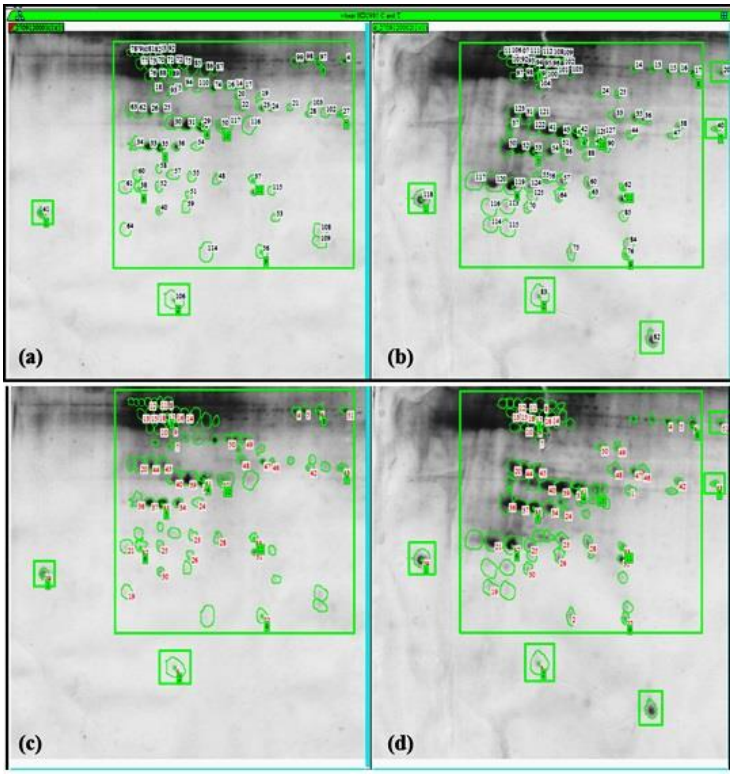

Fig 2. Snap shot of two dimensional electrophoresis (2-DE) gels of control (22°C) and heat shock (42°C, 2 h) treated samples of HD2985 (thermotolerant) cultivar of wheat using IMP7 software (GE Healthcare Life Sciences); (a) & (b) Match-set image showing total number of protein spots identified on the gels, (c) & (d) match-set image showing total number of matched protein spots in both the gels.

sequences observed in control and HS treated samples were used for the identification of protein using online MS homology search tool. Against each peptide, we could observe molecular weight of the protein along with their pI, accession number and further identification of the protein. Most of the proteins were observed to be stress associated proteins like HSP70, ATP synthase, Rubisco small subunit etc.

Peptides and proteins identified in HD2985 wheat cultivar grown under control condition

Shotgun proteomics data generated during this study was characterised using different software's (as listed in materials and methods section). The number of protein groups, total spectral counts, and unique peptides for each replicate was estimated as a function of the LTQ-Orbitrap XL instrument. Ten stress associated peptide sequences were selected from the list of 97 peptides identified in HD2985 (control) and were characterized (Table 4). The peptide sequences observed has molecular weight ranges from 1.2 to 3 kDa with peptide spectral matches ranging from 1 to 20. The peptide sequences were further used for the prediction of respective proteins. An excel sheet with a list of peptides identified in control samples has been depicted in supporting document (Supplementary Table 3). Based on the peptide sequence, the protein groups and their accession number were predicted (Table 5). Most of the proteins were stress associated proteins like HSPs, ATP synthase, calmodulin protein, HSP70 cognate complex (HSC), glyceraldehyde -3- phosphate dehydrogenase (GADPH) etc. Other proteins observed were involved in various other biological pathways like carbohydrate metabolism, signalling pathways, lipid metabolism etc. Our observation is in conformity with the earlier reports (Majoul et al., 2004). It makes us to conclude that plant under control condition execute its normal metabolic pathways along with expression of some of the stress proteins like HSP70 (which plays very important role in protein folding) and calmodulin (which acts as signalling molecule). This is an intelligent mechanism to conserve the total energy by allowing the expression of only those proteins which are required for various metabolic pathways other than protein which are required for defence mechanism in minimal amount. Expression of other stress associated proteins is repressed. The abundance of signalling molecules plays the intermediary role between the expression of normal proteins and stress associated proteins. List of proteins predicted in control samples has been represented in supporting document as excel sheet (Supplementary Table 4).

Peptides and proteins identified in HD2985 wheat cultivar exposed to HS

The raw data from HS treated samples were used for the identification of peptides and we could observe 297 peptides. Ten candidate peptides involved in defence pathway were selected (Table 6). The selected peptides have molecular weight in the range of 1 to 3 kDa. The peptide spectral matches were in the range of 1 to 7. The protein groups and their respective accession number were also predicted based on the peptide sequence (Table 7). A list of peptides identified in treated samples as well as differentially expressed peptides has been represented in supporting document as excel sheet (Table S3).

The sequence of the peptide was used for the prediction of protein group most closely associated. Based on the MASCOT search, we could observe proteins groups involved in various biological pathways like metabolism, defence, signalling, growth and development etc. Many proteins were observed to be involved in defence pathways (Table 7). The percentage of stress associated proteins observed in HS treated samples was far more than that of control sample. The differentially expressed proteins are the candidate proteins involved in thermotolerance pathways and need to be exploited in order to modulate the tolerance level of promising cultivars. Some of the SAPs identified were CPN-60 like chaperonin, HSP70, PWD, luminol binding protein, HSC, ATPase synthase etc. Rollins et al. identified 99 protein spots differentially regulated in response to heat treatment using proteomic tool, 14 of which were regulated in a genotype-specific manner in barley (Rollins et al., 2013). Differentially regulated proteins were predominantly involved in photosynthesis, detoxification, energy metabolism, and protein biosynthesis. Gammulla et al. identified 1100 proteins in one or more temperature treatments in rice and reported more than 400 to be responsive to temperature stress (Gammulla et al., 2011). Proteomic analysis of albumins and globulins present in starchy endosperm of wheat (*Triticum aestivum cv* Recital), at 21 stages of grain development, led to the identification of 487 proteins (Tasleem-Tahir et al., 2012). An excel sheet having list of proteins predicted in treated sample has been depicted in supporting document (Table S4).

Mapping of identified peptides on the Oryza sativa protein database (NCBI RefSeq)

The MS data retrieved from the orbitrap experiment was used for the identification of peptides and proteins in control and HS treated samples of HD2985 cultivar of wheat using *Oryza sativa* protein database (NCBI RefSeq). We could able to identify 317 peptides and 164 protein accession groups in control compared to 592 peptides and 263 protein accession groups in HS treated samples. Proteins with maximum score identified in control were hypothetical protein, GAPDH, heat shock protein70 etc., whereas in case of HS treated samples, Rubisco, Rubisco activase, hypothetical protein, ATP synthase, HSP70 etc. were observed to have maximum score. Some of the other stress associated proteins observed in case of control and HS treated samples were calmodulin, histone h3, betaine aldehyde dehydrogenase, peroxidase, adenosine kinase, AGPase, Photosystem-II protein etc. Many hypothetical proteins were observed with molecular weight in the range of 11 to 180 kDa and are predicted to be possible candidate protein for stress associated tolerance in plants. The number of proteins identified using *Oryza sativa* protein database was more compared to *Arabidopsis thaliana* which makes us to conclude that more extensive bioinformatic data characterization will help us to discover many more significant proteins having prominent role in different abiotic stresses. An excel sheet depicting list of peptides and proteins identified based on the *Oryza sativa* protein database has been represented in supporting document (Supplementary Table 5).

Table 3. Protein spots identified based on the spectrum, mass spectroscopy (MS) data and MASCOT search analysis (Matrix Science).

S.No.	Protein Identification	Accession No.	Organism Name	Spot ID	Sample gel	Mol. Wt. (Da) /pI	Mowse Score	% coverage
1	protein FAR1-RELATED SEQUENCE 5-like	gi|357116466	*Brachypodium distachyon*	66	HD-2985-T	82136/ 8.74	72	12%
2	predicted protein	gi|224140075	*Populus trichocarpa*	84	HD-2985-T	103274/5.72	81	25%
3	hypothetical protein MTR_1g075110	gi|357440995	*Medicago truncatula*	45	HD-2985-T	12854/8.48	58	71%
4	hypothetical protein SORBIDRAFT_02g035390	gi|242045884	*Sorghum bicolor*	118	HD-2985-T	104706/ 5.85	70	7%
5	hypothetical protein SORBIDRAFT_04g004590	gi|242060646	*Sorghum bicolor*	120	HD-2985-T	50892/8.70	57	13%
6	Ribulose bisphosphate carboxylase small chain clone 512; Short=RuBisCO small subunit	gi|132107	*Triticum aestivum*	164	HD-2985-T	13275/5.84	81	53%
7	oxygen-evolving enhancer protein 1, chloroplastic-like isoform 1	gi|357111487	*Brachypodium distachyon*	52	HD-2985-T	34787/ 5.74	92	49%
8	ATP synthase CF1 alpha subunit	gi|14017569	*Triticum aestivum*	15	HD-2985-T	55318/6.11	85	29%
9	ATP synthase CF1 alpha subunit	gi|14017569	*Triticum aestivum*	20	HD-2985-T	55318/6.11	76	26%
10	hypothetical protein CHLNCDRAFT_134230	gi|307107047	*Chlorella variabilis*	16	HD-2985-C	18053/4.96	69	41%
11	oxygen-evolving enhancer protein 1, chloroplastic-like isoform 1	gi|357111487	*Brachypodium distachyon*	35	HD-2985-C	34787/5.74	114	52%
12	predicted protein	gi|168051437	*Physcomitrella patens subsp. patens*	74	HD-2985-C	169790/8.13	73	8%
13	predicted protein	gi|326524408	*Hordeum vulgare subsp. vulgare*	36	HD-2985-C	63985/7.64	69	26%
14	predicted protein	gi|326507050	*Hordeum vulgare subsp. vulgare*	14	HD-2985-C	74755/8.30	61	14%
15	chloroplast fructose-bisphosphate aldolase	gi|223018643	*Triticum aestivum*	32	HD-2985-C	42217/5.94	76	24%

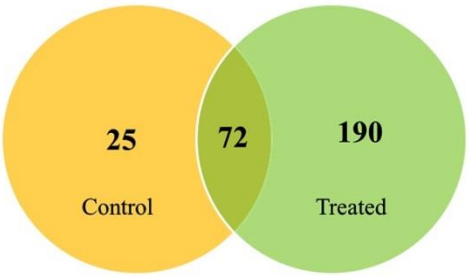

Fig 3. Vienn diagram showing the number of peptides identified in control (22°C) and heat shock (42°C, 2 h) treated samples of HD2985 (thermotolerant) cultivar of wheat by Orbitrap. Twenty five peptides were identified in control sample, whereas 190 and 72 were identified in HS treated sample and in both.

Expression profiling of identified heat stress associated proteins for validation under HS

In present investigation, the proteins identified were classified in to four different group's *i.e.* Heat shock proteins (high and low molecular weight), antioxidant enzyme and stress inducible hypothetical proteins, chaperones and signalling molecules and metabolic pathway associated genes. The accumulation of identified proteins has been correlated with the abundance of transcript level through expression profiling using quantitative real time PCR. The nucleotide sequence of respective genes (based on accession number) were retrieved from the NCBI Gen Bank (HSP90, HSP70, Chl-HSP26, CAT, hypothetical protein-candidate-3174, stress inducible protein, Rubisco activase, calcium dependent protein kinase, calmodulin, ADP-glucose pyrophosphorylase, GADPH and hypothetical protein-candidate-152) and was used for the primer designing using Gene Fischer primer designing software (Supplementary Table 7).

Transcript profiling of heat shock proteins as observed in present investigation showed 2.5, 10 and 28 fold increases in the expression of *HSP90*, *HSP70* and chloroplast *HSP26* in response to heat stress of 42°C for 2 h compared to control (Fig. 4). Huerta et al. observed 50% increases in the expression of *HSP70* over the controls, while the expression increased by 150% over the controls under a water deficit of 50% FC (Huerta et al., 2013).

The expression profiling of antioxidant enzyme and hypothetical protein showed 2.2 (*CAT*), 1.4 (hypothetical protein) and 4.3 (putative stress inducible protein) fold increase in the expression under HS compared to control (Fig. 4). We could also observe 12.2, 5.5 and 3.5 fold increase in the expression of *RCA-I*, *CDPK* and *calmodulin* in HD2985 wheat cultivar under heat stress. The transcript profile of metabolic pathway associated genes showed 0.8, 1.2 and 1.8 fold change in expression of ADP-glucose pyrophosphorylase (*AGPase*), *GAPDH* and hypothetical protein-candidate-152. A high expression of small heat shock protein and chaperones (*RCA*) along with abundance of antioxidant enzyme and other SAPs provide the necessary defence required for the cells to protect itself from elevated temperature. The abundance of signalling molecules like calcium dependent protein kinase (*CDPK*) plays very important role in modulating the expression and activities of other SAPs in response to heat stress. Majoul et al. (2004) reported 24 protein spots to be up-regulated and 19 down-regulated in the non-prolamin, or water soluble, fraction of wheat endosperm under heat stress.

Immunoblot analysis of identified heat stress associated proteins

The monoclonal antibody (anti-HSP70, anti-HSP90 and anti-CDPK synthesized in rabbit; Sigma-Aldrich) were used for the immunoblot analysis in order to confirm the accumulation of stress proteins under elevated temperature in tolerant and susceptible cultivars. Even we could observe many variants of these HSAPs in control and HS treated samples of HD2985 cultivar of wheat (as identified by MALDI-TOF/MS and Orbitrap). Prominent accumulation of HSP70 and CDPK was observed in HS treated samples compared to control (Fig. 5). The accumulation of HSP90 protein was low compared to HSP70. This may be due to the reason that HSP70 has very diverse role to play like protein folding (acts as chaperones) and stress tolerance in cells, whereas HSP90 plays prominent role in signalling, growth and development of tissues. In our earlier observation western blot analysis revealed the presence of 5, 6 and 5 multi-protein chaperone complexes of HSP90 in the range of 95 to 70 kDa at pollination, milky-dough and seed hardening stages in wheat (Kumar et al., 2013a). Grigorova et al. (2011) reported accumulation of HSPs in response to drought and heat stress using immunoblot analysis with anti-HSP70 monoclonal antibodies, anti-HSP110 polyclonal antibodies and anti-α β-crystalline polyclonal antibody. CDPK accumulation was in proportionate with the accumulation of HSPs in response to HS because of its role as secondary signalling molecules. Zou et al. (2010) identified 34 CDPKs from *Arabidopsis* and reported variations in the protein accumulation under drought, ABA and calcium ion treatment. It is the combined activity of all these SAPs which modulates the tolerance mechanism of wheat under heat stress by enhancing the expression of key genes and accumulation of pathways associated proteins, other than protecting the denaturation and aggregation of proteins under elevated temperature.

Materials and Methods

Plant material and stress treatment

Thermotolerant cultivar of wheat (HD2985) was used in this study for the identification of heat-responsive proteins using second generation proteomic tools. Pre-treated seeds (Bavistin 50 DF @ 0.25%) were sown in pots (10"x10") inside regulated chamber (25/20°C day/night with a photoperiod of 16 h, relative humidity of 70% and irradiance level of 250 μmole m^{-2} s^{-1}) at National Phytotron Facility, Indian Agricultural Research Institute (IARI), New Delhi, India. Pots were filled with equal quantity of vermiculite, peat, FYM, sand-mix and irrigation was carried out at regular interval. Six pots in two groups (3 each for control and HS treated) were used for the sowing. One group were exposed to HS of 42°C for 2 h during milky-ripe stage (11.2 on Feekes scale). The leaf samples were collected in triplicates from control and HS treated plants and were immediately freezed in liquid nitrogen for further down-processing.

Table 4. Peptides identified in control (22°C) sample of HD2985 (thermotolerant) cultivar of wheat using Orbitrap.

Sequence	PSMs	Charge	MH+ [Da]	RT [min]	Protein Group Accessions
SVYEPLQTGLIAIDSMIPIGR	1	2	2273.2	105.85	7525018
IINEPTAAAIAYGLDKK	1	2	1788.0	44.02	334185190; 15241849; 30693966; 15223533; 15230534
ATAGDTHLGGEDFDNR	1	2	1675.7	18.69	334185190; 15241849; 15223533; 15230534
MKETAEAYLGK	1	2	1240.6	18.70	30693966
DTDSEEELKEAFR	2	3	1568.7	34.25	30683366
EVAAFAQFGSDLDAATQALLNR	2	2	2308.2	102.64	26557005
VQDLLLLDVTPLSLGLETAGGVMTTLIPR	11	3	3035.7	112.98	15241849
VPTVDVSVVDLTVR	20	2	1498.8	53.45	15229231
GILAADESTGTIGK	4	2	1332.7	25.68	15227981
QFAAEEISSMVLIK	1	2	1565.8	63.56	15223533

*PSM- protein spectrum match; RT- retention time; MH+- singly protonated peptide mass

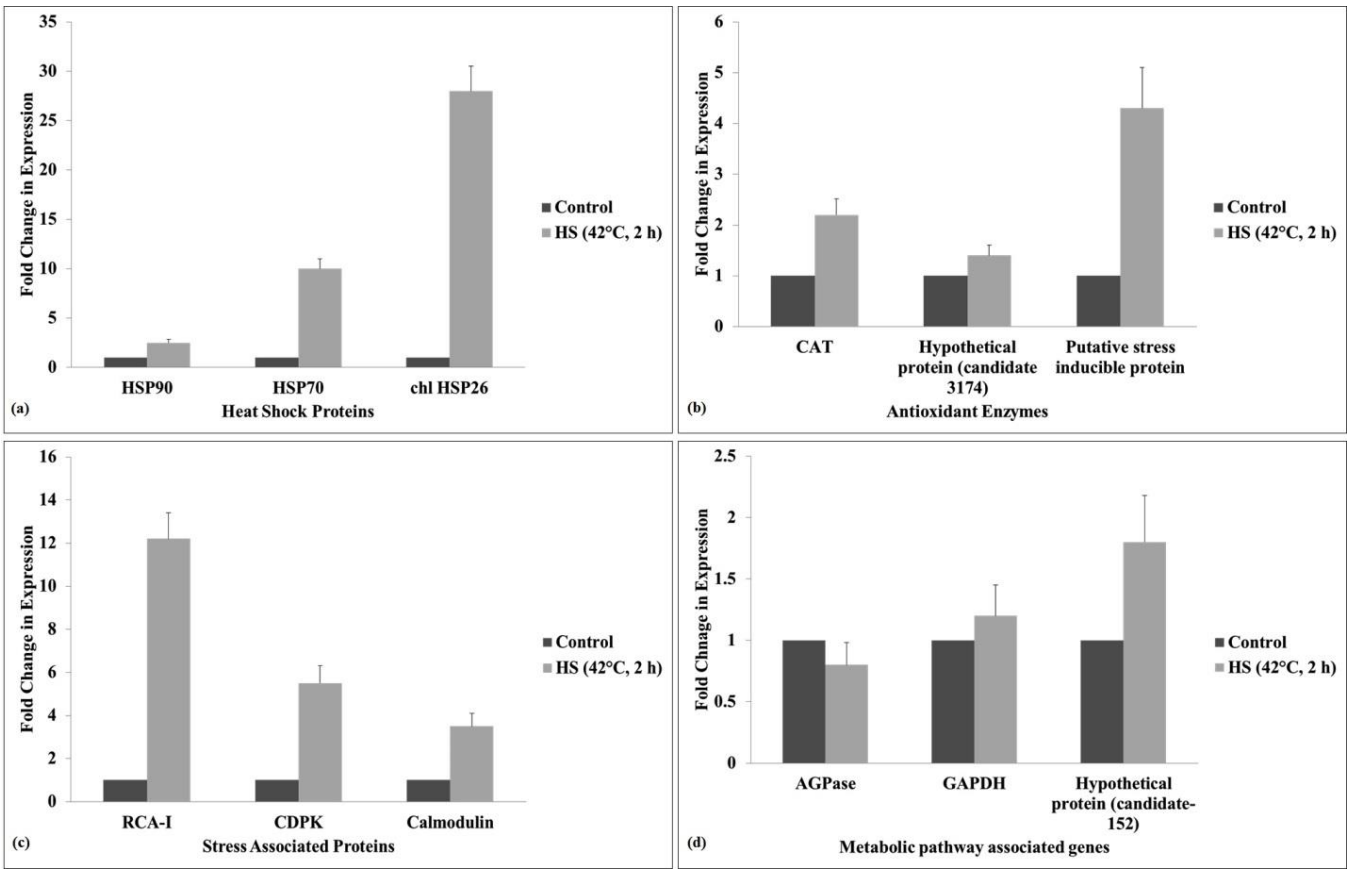

Fig 4. Validation of identified heat stress associated proteins (HSAPs) in HD2985 cultivar of wheat by quantitative real time PCR; primers of heat shock protein 70 (*HSP70*), heat shock protein 90 (*HSP90*), chloroplast heat shock protein 26 (*HSP26*),catalase (*CAT*), hypothetical protein, putative stress protein, rubisco activase (*RCA-I*), calcium dependent protein kinase (*CDPK*), calmodulin, ADP gluco-pyrophosphorylase (*AGPase*) and glyceraldehyde-3-phospahte dehydrogenase (*GAPDH*) were used for expression study in control and HS treated samples; Actin was used as endogenous control gene; relative fold expression was calculated using the method of pfaffl (2002).

Differential protein profiling using two dimensional electrophoresis (2-DE)

Sample preparation

The collected samples (0.5 g) was crushed into fine powder form using liquid nitrogen and homogenized on ice (2 min) in an extraction solution (5.0 ml) consisting of equal volumes of PCI (49% phenol, 49% chloroform, 2% isoamyl alcohol) and NTES (10 mM NaCl, 1 mM EDTA, 1% SDS, 10 mM Tris-HCl, pH 8.5). The phenol phase was recovered by centrifugation (16000 g, 10 min) and re-extracted with an equal volume of NTES; the phenol phase was collected, and diluted with 5 volumes of 0.1 M ammonium acetate in methanol. After overnight incubation (-20°C), the precipitate was centrifuged (16000 g, 10 min) and the pellet was washed with re-suspension buffer (0.1 M ammonium acetate in 1 ml methanol), sonicated on ice for 1 min and then centrifuged . After two more washings with 0.1 M ammonium acetate in methanol (1 ml) and one with acetone (1 ml), the pellet was air-dried. The protein pellet was re-suspended in 0.4 ml sample buffer [9.5 M urea, 2% (v/v) NP-40, 1% (w/v) DTT and 2% (v/v) 3 - 10 Biolyte], centrifuged, and the supernatant was subjected to two-dimensional electrophoresis (2-DE). The protein concentration was measured by Bradford method (Bradford et al., 1976).

Isoelectric focusing and one dimensional electrophoresis (1-DE)

Isoelectric focusing (IEF) was performed using the IEF100 electrophoresis system (Hoefer, USA) and 18 cm SERVA immobilized pH gradient (IPG) blue Strip of 3 – 10 linear pH gradients (SERVA, Germany). The strips were rehydrated overnight in a solution containing 8 M urea, 2% CHAPS, 20 mM DTT, 0.002% bromophenol blue, 2% IPG buffer (pH 3 – 10), and 60 μg of the protein sample. IEF was carried out by applying a voltage of 250 V for 1 h, increasing to 3500 V over 2 h, and holding at 3500 V until a total of 90 kVh was obtained. Following IEF, the strips were equilibrated for 15 min in an equilibration buffer containing 0.05 M Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 20 mM DTT, followed by another 15 min equilibration in the same buffer containing 125 mM iodoacetamide without DTT. The equilibrated strips were applied to vertical SDS–polyacrylamide gels (12.5% resolving, 5% stacking) and sealed with 0.5% agarose in SDS buffer containing bromophenol blue in a slab gel electrophoresis system (ATTO, Japan). The gels were removed and placed in fixative (50% methanol and 10% acetic acid) for overnight. The gels were washed in water for 2 h with three changes and placed in 0.02% sodium thiosulfate solution followed by a brief wash and 0.2% silver nitrate solution for 1 h. After brief water wash the gels were developed in 2% sodium carbonate solution. The gels were stored in 10% acetic acid. The stained gels were scanned using HP Scanjet G4010 (Hewlett-Packard**)**.

Image analysis

The image analysis of the gels was carried out using Image Master 2D Platinum (GE healthcare) version 7.0.6. The software was used to find the total number of spots on the gels and to identify the spots that are differentially expressed between the two samples. First the spots were detected by an algorithm to give identification number (ID), intensity, area and volume to the spots. After spot detection, each and every spot were checked manually for a real spot since the software detects dusts, artifacts, which need to be removed from analysis. The spots that were found at exactly the position across the gels were used as landmarks. The landmarked spots help us in matching the gels perfectly. The spot % volumes was used for calculating the differentially expressed (fold change) spots between the gels. The percent volumes of treated spots divided by the percent volumes of control spot with the same match ID give a ratio that indicates the fold change in expression. Ratio more than 1.5 was considered to be over-expressed and less than 0.5 as under-expressed spots. Spots that did not match in both the gels were considered as unique protein spots. Unique protein spots exclusively present in control or heat shock treated gel were manually QCed before selecting for analysis.

MALDI-TOF-TOF analysis for identification of protein spots

Spot picking and trypsin digestion

The selected spots were manually picked-up one by one using separate 200 µl tip by matching the selected match/spot ID to the actual gel. The selected spots were placed in eppendorf tube containing 10% acetic acid. The silver stained spots were processed for *in gel* digestion by trypsin. In brief, the sample were washed twice with double distil water (DDW) and treated with destaining solution (1:1 solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate) for 30 min. Further the spots were washed in DDW twice followed by treatment with 10 mM DTT at 56°C for 45 min and then with 50 mM iodoacetamide for 30 min in the dark. The spots were then washed with 50 mM ammonium bicarbonate twice and dehydrated with 100% acetonitrile (ACN). The samples were allowed to dry off in the speedvac and then immersed in trypsin solution (0.002% in 50 mM ammonium bicarbonate) and incubated at 37°C with shaking for overnight in a thermo mixer (Eppendorf). After trypsin digestion the released peptides were extracted thrice from the gel by addition of extraction solution (1:1 solution of 100% CAN : 0.1% trifloroacetic acid). The extracted mixture was concentrated by speedvac to dryness.

The dried samples after trypsin digestion were dissolved in TA solvent (Bruker daltonics: mixture of ACN and 0.1% TFA in a volume ratio of 1:2). The matrix solution was prepared by dissolving saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker) in TA solvent. Sample and matrix solution were mixed in the ratio of 1:1 and 1 μl was spotted on to a MTP AnchorTM chip 384 well MALDI target plate (Bruker Daltonics). The spots were then allowed to dry completely (they appear as a thin layer on the surface). The external peptide calibration standard-I (Bruker Daltonics) was also spotted along with the samples in the calibration spots on the plate.

Samples were analyzed on a MALDI-TOF-TOF mass spectrometer (Ultraflex III, Bruker Daltonics) using an accelerating voltage of 25 kV, 25% laser power and 200 spectra/sec speed for the Peptide Mass Fingerprint (PMF) mode. The sample spectrum (m/z) was obtained after calibration of the instrument with the external calibration standards in Flex control software (Bruker Daltonics). The Flex

Table 5. Proteins identified in control (22°C) sample of HD2985 (thermotolerant) cultivar of wheat based on homology search using the protein database of *Arabidopsis thaliana* (NCBI RefSeq).

Accession	Description	Score	Coverage	Unique Peptides	Peptides	PSMs	AAs	MW [kDa]	Calc pI
7525018	ATP synthase CF1 alpha subunit [*Arabidopsis thaliana*]	3.54	4.14	1	1	1	507	55.3	5.25
334185190	protein heat shock protein 70-3 [*Arabidopsis thaliana*]	96.42	20.03	1	10	21	649	71.1	5.07
15230534	heat shock protein 70-4 [*Arabidopsis thaliana*]	48.63	16.77	1	8	11	650	71.1	5.25
30693966	Luminal-binding protein 2 [*Arabidopsis thaliana*]	15.94	4.57	1	3	4	613	67.4	5.29
30683366	calmodulin 5 [*Arabidopsis thaliana*]	16.80	26.55	2	2	4	113	12.9	4.32
26557005	ATPase subunit 1 [*Arabidopsis thaliana*]	9.83	4.34	1	1	2	507	54.9	6.61
15241849	heat shock 70kDa protein 1/8 [*Arabidopsis thaliana*]	158.19	26.57	2	12	33	651	71.3	5.12
15229231	glyceraldehyde-3-phosphate dehydrogenase, cytosolic [*Arabidopsis thaliana*]	128.76	22.78	4	4	30	338	36.9	7.12
15227981	fructose-bisphosphate aldolase, class I [*Arabidopsis thaliana*]	13.20	3.91	1	1	4	358	38.4	7.39
15223533	heat shock protein-70 cognate protein [*Arabidopsis thaliana*]	42.14	15.56	1	7	9	617	68.3	5.35

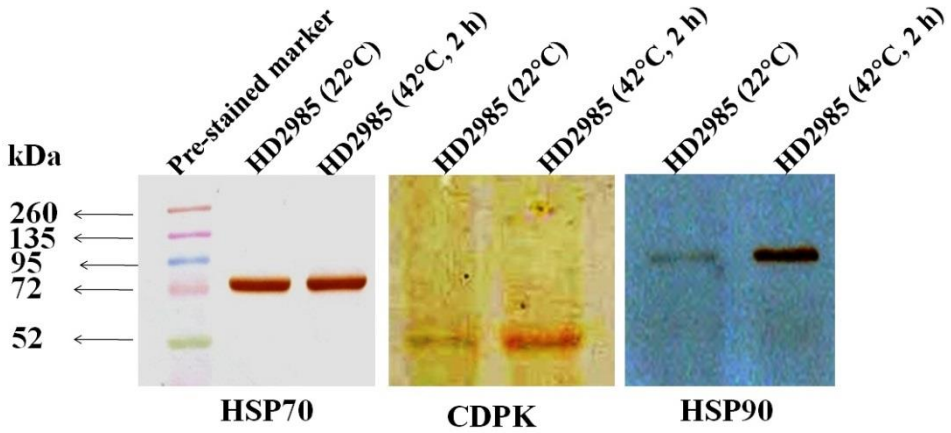

Fig 5. Immunoblot analysis of control (22°C) and heat shock (42°C, 2 h) treated samples of HD2985 cultivar of wheat using monoclonal antibodies of heat shock protein 70 (HSP70), heat shock protein 90 (HSP90) and calcium dependent protein kinase (CDPK); Anti-HSP70, Anti-HSP90 and Anti-CDPK were synthesized in rabbit; pre-stained protein marker was used for analysing the molecular weight of the respective band.

Table 6. Peptides identified in heat shock (42°C, 2 h) treated sample of HD2985 (thermotolerant) cultivar of wheat using orbitrap.

Sequence	PSMs	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	Protein Group Accessions
SVYEPLQTGLIAIDSMIPIGR	4	3	2273.24	6.42	98.77	7525018
AAVEEGIVPGGGVALLYASK	1	2	1901.04	3.69	53.64	334184654
EIAEAYLGTTIKNAVVTVPAYFNDSQR	1	3	2970.50	-6.82	90.10	334185190
LQDLLNLVK	1	2	1055.65	-0.05	29.77	79485345
DILLLDVAPLTLGIETVGGVMTK	2	3	2368.36	9.80	110.89	30693966
AMELDDEDISYLTNR	1	2	1784.80	2.27	49.10	30682109
GPILLEDYHLLEK	1	3	1539.84	-2.54	50.78	18394890
VQDLLLLDVTPLSLGLETAGGVMTTLIPR	4	3	3035.73	8.87	111.32	15241849
EQVFSTYSDNQPGVLIQVFEGER	3	3	2642.29	5.55	73.96	15241847
KQDITITGASTLPK	1	2	1472.83	-2.15	22.27	15233779
VQDLLLLDVTPLSLGLETAGGVMTVLIPR	2	3	3033.76	9.93	111.58	15230534
EADVDGDGQINYEEFVK	7	2	1927.86	0.80	43.69	15229784
VGAATETELEDR	1	2	1290.62	0.16	15.09	15226314
FSDASVQSDMK	2	2	1214.54	0.08	17.05	15223533
GPIQITWNYNYGAAGK	1	2	1752.87	1.16	49.87	15224319

*****PSM- protein spectrum match; RT- retention time; MH+- singly protonated peptide mass

analysis 3.3 software (Bruker Daltonics) was used to extract and process the peptide mass peaks from the spectrum. The post analysis processed spectrum was used for searching protein using different databases available on public domain.

In silico protein identification

The measured and calibrated tryptic peptide masses were transferred through MS BioTool (Version 3.2, Bruker daltonics) as inputs to search against the non-redundant NCBI (NCBInr) database using MASCOT 2.2 (Matrix Science) search engine. Search parameters were as follows: Taxonomy: Viridiplantae (green plants); trypsin cleavage; allow up to one missed cleavage; peptide mass tolerance 0.2 Da; fixed modification: carbamidomethyl (C); variable modification: oxidation (M). Protein identifications were accepted if they had greater than 95% probability as represented by the mascot scores in the mascot result page.

Label-free identification of proteins using orbitrap

Sample preparation

The collected plant sample (100 mg) was used for the protein extraction using plant protein extraction kit (Sigma). The plant tissue has lot of Rubisco in it. So to enrich low abundant proteins the plant Rubisco was removed through the process of Immunocapture using IgY SEPPRO RUBISCO spin columns (Sigma). The collected flow through was processed through 2D clean up kit (GE Health Care) as per the manufacturers instruction.

Trypsin digestion and separation of peptides

About 100 µg of protein extract from each sample was processed for trypsin digestion using the protocol as mentioned above. The speedvac trypsin digested samples were dissolved in 0.5% acetic acid for separation on nanoflow HPLC instrument (Easy nLC Proxeon Biosystems) now known as Thermo Fisher Scientific coupled to a mass spectrometer. The chromatography columns were packed in-house with ReproSil-Pur C18-AQ 3_m resin in buffer A (0.5% acetic acid) with dimensions of 10 cm long, 75 mm inner diameter. The digested peptide mixture dissolved in 0.5% acetic acid was loaded onto the column and was separated with a linear gradient of 0-30% buffer B (100% Acetonitrile and 0.5% acetic acid) at a flow rate of 250 nl min^{-1} over a period of 90 min and total analysis time of 120 min. The elution profile was monitored by the TIC (Total Ion Chromatography) of the mass spectrometer.

MS and MSMS (MS2) analysis

Peptide fractions separated on the nLC column were analyzed on a hybrid linear ion-trap/Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Scientific) equipped with a nano flow high-performance liquid chromatography system (Thermo Scientific). The separated peptides were ionized using electrospray ionization at a heated capillary temperature of 275°C, and measured in the orbitrap mass spectrometer. The LTQ Orbitrap Velos was operated in data dependent mode to automatically switch between MS and MS2 acquisitions (Supplementary Table 6). Survey full scan MS spectra (*m*/*z* 300–1150) were acquired in the Orbitrap with a MS resolution of 60,000 and MSMS resolution of 15000.

The MSMS fragmentation was achieved by HCD (higher energy collisional dissociation cell) mode in data dependent manner (top 20) with an ion selection threshold of 5,000 counts, isolation window of 4.0 Da, an activation time of 0.1 ms, and a normalized collision energy of 40% were used. Peptides with unassigned charge states, as well as with charge state less than 3 were excluded from fragmentation (Supplementary Table 6). Fragment spectra were acquired in the Orbitrap mass analyzer. The mass spectra were acquired and the parameters were controlled by X caliber 2.1 (Thermo) software. The data output were analyzed using SEQUEST search algorithm against *A. thaliana* protein database (NCBI RefSeq). Trypsin was used as the enzyme allowing for up to 1 missed cleavage. The mass tolerances for precursor ion and fragment ions were set to 20 ppm and 0.1 Da, respectively. Carbamidomethyl and oxidation of methionine were allowed as constant and variable modifications respectively. The false discovery rate (FDR) was set to 0.01 for proteins and peptides, which should have a minimum length of 6 amino acids. Peptide and proteins were identified with >95.0% probability and

Table 7. Protein identified in heat shock (42°C, 2 h) treated sample of HD2985 (thermotolerant) cultivar of wheat based on homology search using the protein database of *Arabidopsis thaliana* (NCBI RefSeq).

Accession	Description	Score	Coverage	Unique Peptides	Peptides	PSMs	AAs	MW [kDa]	Calc. pI
334184654	chaperonin CPN60-like 1 [*Arabidopsis thaliana*]	4.42	3.45	1	1	1	580	61.4	6.3
334185190	protein heat shock protein 70-3 [*Arabidopsis thaliana*]	215.81	35.75	2	15	48	649	71.1	5.1
79485345	phosphoglucan, water dikinase [*Arabidopsis thaliana*]	3.262	0.7	1	1	1	1278	144.7	8.5
30693966	Luminal-binding protein 2 [*Arabidopsis thaliana*]	48.8	14.03	5	6	12	613	67.4	5.3
30682109	putative stress-inducible protein [*Arabidopsis thaliana*]	3.947	2.83	1	1	1	530	60.4	6.1
18394890	catalase 1 [*Arabidopsis thaliana*]	4.15	2.64	1	1	1	492	56.7	7.4
15241847	heat shock protein 70 [*Arabidopsis thaliana*]	194	38.59	2	16	43	653	71.3	5.1
15241849	heat shock 70kDa protein 1/8 [*Arabidopsis thaliana*]	284.6	37.79	2	17	68	651	71.3	5.1
15233779	chloroplast heat shock protein 70-1 [*Arabidopsis thaliana*]	5.922	1.95	1	1	1	718	76.5	5.2
15230534	heat shock protein 70-4 [*Arabidopsis thaliana*]	179.2	40.46	4	17	41	650	71.1	5.2
15229784	calmodulin 7 [*Arabidopsis thaliana*]	37.31	31.54	3	3	9	149	16.8	4.3
15226314	chaperonin-60 alpha [*Arabidopsis thaliana*]	3.337	2.05	1	1	1	586	62.0	5.2
15223533	heat shock protein-70 cognate protein [*A. thaliana*]	179.1	35.17	1	14	40	617	68.3	5.4
15224319	putative chitinase [*Arabidopsis thaliana*]	4.121	5.69	1	1	1	281	30.0	9.4
7525018	ATP synthase CF1 alpha subunit [*Arabidopsis thaliana*]	22.64	6.71	2	2	5	507	55.3	5.2

*AAs- amino acid sequence; PSM- protein spectrum match; MW- molecular weight

proteins identified with at least 2 identified peptides were retained.

Validation of identified heat stress associated proteins (HSAPs) genes

The identified heat-responsive proteins in present investigation were subjected to validation by profiling their expression pattern under differential HS in tolerant and susceptible cultivars using quantitative real time PCR. The collected samples were used for the total RNA isolation using the Trizol method (Invitrogen, UK) and the quantification was done using Picodrop (Thermo Scientific, USA). RNA integrity was checked on 1.2% agarose gel. First strand cDNA synthesis was done using oligo dT primer (RevertAid^{TM} H minus First Strand cDNA synthesis kit (Thermo Scientific, USA). First-strand cDNA was prepared from 1 µg/µl concentration of RNA. Primers (HSP90, HSP70, Chl-HSP26, CAT, hypothetical protein-candidate-3174, stress inducible protein, Rubisco activase, calcium dependent protein kinase, calmodulin, ADP-glucose pyrophosphorylase, GADPH and hypothetical protein-candidate-152) for quantitative RT-PCR reactions were designed from the conserved sequence of the respective genes retrieved from the Gen Bank using Gene Fischer primer designing software (Table 2). Expression analysis was performed using three biological and two technical replicates. Quantitative RT-PCR was performed in 20 µl reaction using gene specific primers (10 mM) 0.4 µl each, 1 µl of cDNA (generated from 1 µg/µl concentration of RNA) as template and the Fast SYBR Green PCR Master Mix (Kappa Biosystems). Reactions were performed on the CFX96 platform (Bio Rad, UK). The thermal cycle for qRT-PCR was: 95°C for 2 min, followed by 39 cycles consisting of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s followed by plate read. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis (65° to 95°C with an increment of 0.5°C in 10 s) and agarose gel electrophoresis on a 2% agarose gel. The expression levels of wheat Actin gene were used as endogenous control gene for the normalization of C_t value. Data analysis was performed using software provided by Bio Rad, UK. The comparative C_t ($2^{-\Delta\Delta Ct}$) method was used to calculate the fold change in the expression of the genes compared to control (Pfaffl et al., 2002).

Western blot analysis of identified heat stress associated proteins (HSAPs)

Leaves collected from control and HS treated HD2985 cultivar (0.3 g) were ground to fine powder using liquid nitrogen. The fine powder was transferred immediately to eppendorf tube containing 1 ml extraction buffer (50 mM phosphate, pH 7.0, 0.2% (v/v) Triton-X-100, 7 mM β-mercaptoethanol and 5 mM ascorbic acid). The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant collected was used for the SDS-PAGE with a discontinuous buffer system (Laemmli et al., 1970). Concentrations of proteins were determined according to the Bradford protocol (Bradford, 1976). 15 µg of protein samples were separated electrophoretically on SDS - PAGE and transferred onto polyvinylidene fluoride (PVDF) micro-porous membranes using the semi-dry iblotter (Invitrogen, UK). The membrane was incubated in blocking buffer [1 g BSA in 20 ml TBS (pH 7.6), 30 µl Tween20 and make up volume to 30 ml with TBS] for overnight at 4°C with shaking. Membrane was washed in TBST [0.1% Tween20 (3 times) for 10 min each]. Further the membrane was incubated with primary monoclonal antibody (anti-HSP70, anti-HSP90 and anti-CDPK for separate experiment) in TBS for 1 hour at room temperature with gentle shaking (in dilution of 1: 4,000). Again, it was washed in TBST [0.1% Tween20 (3 times) for 10 min each]. Further, the membrane was incubated with secondary antibody (peroxidase-conjugated goat anti-mouse IgG) following the manufacturer's instruction (in dilution of 1:4000); 1 hour at room temperature. Washing step was carried out in TBST [0.1% Tween20, (3 times) for 10 min each]. Membrane was transferred to a shallow tray. Add 15 µl H_2O_2 (30%) to 10 ml of 0.05% 3, 3'-diaminobenzidine (DAB) in TBS, mix well immediately; the DAB was pour to the membrane, incubate at room temperature with gentle shaking in the dark if possible. When the bands are of the desired intensity (2-5 min), the filter was washed briefly in water and in TBS.

Conclusion

In wheat, the information on heat-responsive proteins is limited and the mechanism associated with the tolerance has not been fully characterised. Here, we have identified 47 (control) and 38 (HS) unique protein spots with 19 differentially expressed proteins in HD2985 wheat cultivar using 2-DE. Using orbitrap (label-free) analysis, we could able to identify 97 peptides, 60 proteins (control) and 262 peptides, 135 proteins accession group (HS) using *Arabidopsis* protein database. Similarly, 317 peptides, 164 proteins (control) and 592 peptides, 263 protein accession groups (HS) were identified using *Oryza sativa* protein database. Most of the proteins identified were involved in stress associated pathways, metabolic pathways, signalling pathways etc. We could observe high transcript of HSPs, chaperones, stress predicted protein, signalling molecules and low transcript of metabolic pathway associated genes in wheat under HS. Immunoblot analysis also validates the accumulation of HSAPs and confirmed our transcript profiling results. These identified heat-responsive proteins can be further characterised for understanding the mechanism of heat stress tolerance and their respective genes can be used as marker for back-cross breeding program. Potential candidate genes can be manipulated for the development of climate smart wheat crop.

Acknowledgment

The authors would like to express their gratitude to Dr. H. S. Gupta, Director, Indian Agricultural Research Institute (IARI), New Delhi, India for providing the logistic support in order to take up this research work. This work was supported by the Indian Council of Agriculture Research (ICAR), Government of India (GOI) under National Initiative for Climate Resilient Agriculture (NICRA) project (TG-10/3079).

References

Abdalla KO, Rafudeen MS (2012) Analysis of the nuclear proteome of the resurrection plant (*Xerophyta viscosa*) in response to dehydration stress using iTRAQ with 2DLC and tandem mass spectrometry. J Proteomics. 75(8):2361-2374

Bazargani MM, Sarhadi E, Bushehri AA, Matros A, Mock HP, Naghavi MR, Hajihoseini V, Mardi M, Hajirezaei MR,

Moradi F, Ehdaie B, Salekdeh GH (2011) A proteomics view on the role of drought-induced senescence and oxidative stress defence in enhanced stem reserves remobilization in wheat. J Proteomics. 74(10):1959-1973

Boye JI, Achouri A, Raymond N, Cleroux C, Weber D, Koerner TB, Hucl P, Patterson CA (2013) Analysis of glabrous canary seeds by ELISA, mass spectrometry and western blotting for the absence of cross-reactivity with major plant food allergens. J Agric Food Chem. 61(25):6102–6112

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248-254

Dani V, Simon WJ, Duranti M, Croy RR (2005) Changes in the tobacco leaf apoplast proteome in response to salt stress. Proteomics. 5(3):737-745

Gammulla CG, Pascovici D, Atwell BJ, Haynes PA (2011) Differential proteomic response of rice (*Oryza sativa*) leaves exposed to high-and low-temperature stress. Proteomics. 11(14):2839-2850

Grigorova B, Vaseva I, Demirevska K, Feller U (2011) Combined drought and heat stress in wheat: changes in some heat shock proteins. Biol Plant. 55(1):105-111

Guo G, Ge P, Ma C, Li X, Lv D, Wang S, Ma W, Yan Y (2012) Comparative proteomic analysis of salt response proteins in seedling roots of two wheat varieties. J Proteomics. 75(6):1867-1885

Haddad TM, Bancel E, Martre P, Triboi E, Branlard G (2013) Effect of short heat shocks applied during grain development on wheat (*Triticum aestivum* L.) grain proteome. J Cereal Sci. 57(3):486-495

Huerta C, Freire M, Cardemil L (2013) Expression of hsp70, hsp100 and ubiquitin in *Aloe barbadensis* Miller under direct heat stress and under temperature acclimation conditions. Plant Cell Rep. 32(2):293-307

Jagadish SV, Muthurajan R, Oane R, Wheeler TR, Heuer S, Bennett J, Craufurd PQ (2010) Physiological and proteomic approaches to address heat tolerance during anthesis in rice (*Oryza sativa* L.). J Exp Bot. 61(1):143-156

Kumar RR, Goswami S, Sharma SK, Gadpayle KA, Kumar N, Rai GK, Singh M, Rai RD (2012) Protection against heat stress in wheat involves change in cell membrane stability, antioxidant enzymes, osmolyte, H_2O_2 and transcript of heat shock protein. Int J Plant Physiol Biochem. 4(4):83-91

Kumar RR, Goswami S, Sharma SK, Singh K, Gadpayle KA, Singh SD, Pathak H, Rai RD (2013a) Differential expression of heat shock protein and alteration in osmolyte accumulation under heat stress in wheat. J Plant Biochem Biotechnol. 22(1):16-26

Kumar RR, Sharma SK, Goswami S, Singh GP, Singh R, Singh K, Pathak H, Rai RD (2013b) Characterization of differentially expressed stress-associated proteins in starch granule development under heat stress in wheat (*Triticum aestivum* L.). Indian J Biochem Biophys. 50(2):126-138

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259):680-685

Laino P, Shelton D, Finnie C, De Leonardis AM, Mastrangelo AM, Svensson B, Lafiandra D, Masci S (2010) Comparative proteome analysis of metabolic proteins from seeds of durum wheat (*cv*. Svevo) subjected to heat stress. Proteomics. 10(12):2359-2368

Li W, Zhang C, Lu Q, Wen X, Lu C (2011) The combined effect of salt stress and heat shock on proteome profiling in *Suaeda salsa*. J Plant Physiol. 168(15):1743-1752

Ling HQ, Zhao S, Liu D, Wang J, Sun H et al. (2013) Draft genome of the wheat A-genome progenitor *Triticum urartu*. Nature. 496:87–90

Liu T, Zhang L, Yuan Z, Hu X, Lu M, Wang W, Wang Y (2013) Identification of proteins regulated by ABA in response to combined drought and heat stress in maize roots. Acta Physiol Plant. 35(2):501-513

Majoul T, Bancel E, Triboi E, Ben Hamida J, Branlard G (2004) Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from non-prolamins fraction. Proteomics. 4(2):505-513

Makarov A, Denisov E, Lange O, Horning S (2006) Dynamic range of mass accuracy in LTQ Orbitrap hybrid mass spectrometer. J Am Soc Mass Spectrom. 17(7):977-982

Oliveira E, Amara I, Bellido D, Odena MA, Domínguez E, Pages M, Goday A (2007) LC-MSMS identification of *Arabidopsis thaliana* heat-stable seed proteins: enriching for LEA-type proteins by acid treatment. J Mass Spectrom. 42(11):1485-1495

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30(9):e36

Rollins JA, Habte E, Templer SE, Colby T, Schmidt J, Von Korff M (2013) Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.). J Exp Bot. 64(11):3201-3212

Sairam RK, Srivastava GC, Saxena DC (2000) Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. Biol Plant. 43(2):245-251

Tasleem-Tahir A, Nadaud I, Chambon C, Branlard G (2012) Expression profiling of starchy endosperm metabolic proteins at 21 stages of wheat grain development. J Proteome Res. 11(5):2754-2773

Vensel W, Harden L, Tanaka C, Hurkman W, Haddon W (2002) Identification of wheat endosperm proteins by MALDI mass spectrometry and LC-MS/MS. J Biomol Tech. 13(3):95

Zou JJ, Wei FJ, Wang C, Wu JJ, Ratnasekera D, Liu WX, Wu WH (2010) Arabidopsis calcium-dependent protein kinase CPK10 functions in abscisic acid-and Ca^{2+}-mediated stomatal regulation in response to drought stress. Plant Physiol. 154(3):1232-1243