Elucidating stress proteins in rice (*Oryza sativa* L.) genotype under elevated temperature: a proteomic approach to understand heat stress response

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Abstract Rice is one of the widely consumed staple foods among the world's human population. Its production is adversely affected by high temperature and is more pronounced at flowering stage. Elucidating elevated temperature stress-related proteins as well as associated mechanisms is inevitable for improving heat tolerance in rice. In the present study, a proteomic analysis of heat-sensitive rice genotype, IET 21405 was conducted. Two-dimensional electrophoresis (2-DE) and MALDI-TOF/MS-based proteomics approaches revealed a total of 73 protein spots in rice leaf. The protein profiles clearly indicated variations in protein expression between the control and heat treated rice genotypes. Functional assessment of 73 expressed proteins revealed several mechanisms thought to be involved in high temperature including their putative role in metabolism, energy, protein synthesis, protein transport/storage, etc. Besides these, some proteins are expected to involve in photosynthesis, tricarboxylic acid

(TCA) cycle, glycolysis and other proteins for energy production. The proteins identified in the present study provide a strong basis to elucidate gene function of these proteins and to explain further the molecular mechanisms underlying the adaptation of rice to high temperature stress.

Keywords Rice · High temperature stress · Stress proteomics · 2-D Electrophoresis · MALDI-TOF-MS analysis

Introduction

Temperature is one of the crucial abiotic factors, which determine the plant growth and development, due to continuous increase in the concentration of green house gases viz. CH₄, CO₂, CFCs, N₂O; the overall global temperature is increasing continuously in mean surface air temperature

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from 1.5 to 5.4 °C (IPCC 2014) with an increased variability. This increase in global temperature severely affects the crop cultivation and productivity (Kim et al. 2015). It also shortens the life cycle of the cereal crop plants by reducing the duration of developmental phases. Rice is one of the most important examples of cereal crops as it is the staple food crop and major cereal crop consumed by humans. It has some ability to endure high temperature as it is native of tropical and subtropical areas. However, the growth of normal rice can be adversely affected in temperatures above a certain threshold (Kaneko et al. 2016; Kim et al. 2015). In addition, grain yield of rice cannot be achieved due to various environmental constraints such as temperature, drought, salinity, osmotic stress, nutrient imbalance, etc. Among them, temperature is one of the major yield-limiting constraints which is crucial for rice crop during flowering and grain filling stages (Rang et al. 2011; Cho and Oki 2012). The recognition of the genes and proteins that are responsible for various abiotic stresses, particularly heat stress is an indispensable path towards understanding the molecular mechanisms underlying the stress response (Kaneko et al. 2016). To understand the mechanisms of heat tolerance in rice, proteomic study is essential in response to high temperature. From more than three and half decade, the MALDI-TOF is been utilized to assess proteomics biology. It is been used to study the crucial pathway for elevated temperature stress responsiveness and tolerance (Huang et al. 2012). 2-DE in combination with MS allows rapid and reliable protein identification (Klose 2009). In recent years, proteomic approaches are used for the systematic and functional study of the responses to a wide range of abiotic stresses including heat stress. Several genes and proteins that respond to elevated temperature have already been identified (Liao et al. 2012; Mittal et al. 2012), which provides an understanding about molecular mechanisms of this important crop. During the ripening period, elevated temperature affects quality due to impaired deposition and the transformation of storage materials (viz. starch content and protein accumulation) in rice (Zhong et al. 2012). Accumulation of heat shock proteins (HSPs) under the control of heat stress transcription factors (HSFs) is known to play a major role in heat stress response (HSR) and in acquiring thermo-tolerance in plants and other organisms. HSPs functionally act as molecular chaperons and thought to repair as well as aid in the renaturation of stress-damaged proteins (Hasanuzzaman et al. 2013). Hsps/chaperones are responsible for stabilizing proteins and membranes, protein folding and can also assist in protein refolding under elevated temperature. They play a crucial role in protecting plants against stress by re-establishing normal protein conformation and thus cellular homeostasis (Wang et al. 2004).

Recently, proteomic approaches have been widely applied to the systematic study of plant responses in reference to a wide range of abiotic stresses (Kaneko et al. 2016; Kim et al. 2015; Neilson et al. 2010). Advances in the proteome characteristics and identification of HSPs in rice will assist in understanding the regulatory network of this valuable crop. Although the first half of the ripening period of rice is very sensitive to elevated temperature stress (Li et al. 2011), few functional proteins involved in response to elevated temperature have been identified (Timperio et al. 2008; Neilson et al. 2010). The purpose of this study was to understand the role of various heat shock proteins under elevated temperature stress in rice genotype. Such a study would have utility in identifying role of these critical proteins during temperature stress in rice plant.

Materials and methods

Experimental site

A field experiment was laid out in *kharif* seasons (June–November) of 2012 and 2013 at Norman E. Borlaug Crop Research Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, US Nagar (Uttarakhand), India. Geographically, the site lies in Tarai plains about 30 km southwards of the foothills of the Shivalik range of the Himalayas at 29°3'N latitude, 79°29'E longitude and at an altitude of 243.8 m above the mean sea level. The experimental plot (typical haplindoll) had a loam texture, 7.0 pH, 0.278 dS m⁻¹ EC at 25 °C, 10.3 g organic C kg⁻¹.

Plant materials and treatments

The seeds of eleven rice genotypes was obtained from the Directorate of Rice Research, Hyderabad and grown with three replications in a randomized block design (RBD). The seeds were sown in June, with an aim to impose extremely hot temperatures during the vegetative stage and flowering stage. The seeds of all 11 genotypes were sown on 13 June 2011. Twenty-one-day-old seedlings were transplanted in three rows and each row has 15 plants. Intra- and inter-row distance was maintained at 10 and 20 cm, respectively, with a 10-cm gap between each entry. In main experimental plot (27 × 3 m) Nitrogen (urea), phosphorus (single superphosphate) and potassium (muriate of potash) were applied at the rate of 120, 40 and 60 kg ha⁻¹ with complete P and K fertilizer applied as a basal dose while N was applied in three splits: 50% applied a day before transplanting, a second split of 25% applied at active tillering and the remaining 25% applied when a majority of the genotypes were at the booting stage. The heat stress treatment was given during flowering. The

terminal heat stress was imposed in one block by covering with 0.1 mm thick UV transparent polyvinyl chloride film. For this purpose a temporary poly house was made under field condition during the reproductive stage. Another block which was not covered with polythene sheet was treated as a control. Maximum and minimum temperatures were recorded daily with the help of an automatic thermometer inside and outside the PVC tunnel. The mean temperature inside the tunnel was about 12 °C during the highest peak of flowering stage (Table 1). The trial of 11 genotypes has been done in 2011 and 2012. Out of these we have screen out the sensitive one genotype (IET 21405) for the 2-DE gel electrophoresis (2-DE) analysis (Kumar et al. 2015).

Protein extraction and proteome analysis

Extraction of total proteins from rice leaves at flowering stage was carried out according to the method illustrated Reza et al. 2005. Lyophilized protein sample was sent to Sandor Proteomics Pvt Ltd, Hyderabad for 2-DE. In silico, study of 2-DE gel was done on the basis of isoelectric point (*pI*) and molecular weight (M_w) (Li et al. 2013). MALDI-TOF analysis was done at Sandor Proteomics Pvt Ltd, Hyderabad using Bruker Daltonics—Ultraflex™ III Mass Spectrometer with spectra internally calibrated using trypsin auto-digestion products. The obtained peptide masses were searched on the Mascot™ Peptide Mass Fingerprint database (Matrix Science). The data obtained were used to determine the proteins using the Mascot search tool (www.matrixscience.com).

Principal component analysis (PCA)

The protein spot intensities were analyzed using PDQuest and imported into the statistical package for social science (SPSS) version 16. SPSS was used to perform PCA based on protein spot *pI* as PC I, molecular weight (*D*) as PC II and their expression values as PC III (Fig. 3).

Results and discussion

A quantitative analysis by 2-DE revealed a total of 73 differentially expressed protein spots (Fig. 1). A pH gradient from 4.0 to 7.0 was used to analyze heat stress protein expression under elevated temperature. Principal component analysis (PCA) revealed that heat stress expressed proteins comprise the patterns with a high density of spots in the low molecular weight acidic range (Fig. 2). Some of the identified proteins were shown either as unknown and or hypothetical. Among the identified protein spots, 20 spots were found to be up-regulated while 53 spots were down-regulated. It is observed that most of the heat stress-related proteins have a molecular weight ranges from ~6 to 114 kDa. Four protein spots were selected randomly for MALDI-TOF-MS analysis, remaining 69 were analyzed by 2-DE gel images manually (Suyal et al. 2014). All the spots are summarized with their *pI* and M_w in Table 2.

The expressed proteins were classified according to their functions (Zhang et al. 2005). Out of these, 72% spots were mainly related to defense, 10% protein synthesis and modification, 9% intracellular traffic and transporter, 5% signal transduction and metabolism, 2% cell structure and 2% energy (Fig. 3).

Table 1 Temperature recorded in two successive years

Month	Date	2010				2011			
		Ambient temperature		Temperature in tunnel		Ambient temperature		Temperature in tunnel	
		Max	Min	Max	Min	Max	Min	Max	Min
Aug–Sep	27-02	32.0	25.0	43.3	25.1	34.4	25.9	39.3	26.3
Sep	03-09	30.7	24.9	42.2	26.2	32.6	24.7	44.5	25.7
Sep	10-16	30.8	23.9	44.6	24.3	32.5	24.4	43.8	26.7
Sep	17-23	27.8	23.5	41.5	24.7	31.6	23.2	39.2	25.8
Sep	24-30	31.7	21.9	42.5	24.5	31.5	22.2	43.7	24.8
Oct	01-07	32.3	21.1	39.2	25.7	31.6	21.4	43.1	23.4
Oct	08-14	31.6	19.4	41.7	22.4	33.3	19.4	43.1	23.0
Oct	15-21	32.6	20.6	40.0	21.2	31.6	15.9	42.0	16.4
Oct	22-28	31.3	15.2	39.2	16.5	30.8	14.4	41.0	16.2
Oct–Nov	29-04	29.8	14.5	36.4	15.1	28.5	14.1	39.4	15.4

Fig. 1 2-DE profile of rice genotype under control (a) and high temperature stress (b). Differentially expressed proteins identified by MALDI-TOF analysis are numbered from 1 to 4 with a rectangle

Fig. 2 Principal component score plot for expression profiling of the proteins at high temperature in rice genotype. SPSS was used to perform PCA based on protein spot pI as PC I molecular weight (D) as PC II and their expression values (proteins those were exclusively present and those up-regulated under heat stress given the values +1 whereas proteins those were completely absent and those down-regulated under heat stress given the values -1) as PC III

72% of the identified proteins were found to belong to defense and stress response. HSPs are chaperones, which play a crucial role in protecting plants against stress by re-establishing normal protein conformation. Plant stress responses require both protective measures that reduce or restore stress-inflicted damage to cellular structures and mechanisms that efficiently remove damaged and toxic macromolecules, such as misfolded and damaged proteins. It was reported that NBR1, the first identified plant autophagy adaptor with an ubiquitin-association domain, plays a critical role in plant stress tolerance by targeting stress-induced, ubiquitinated protein aggregates for degradation by autophagy (Zhou et al. 2014).

The glycine-rich RNA-binding proteins (GRPs) are found ubiquitously in plants. These proteins contain a glycine-rich region at the C-terminus and one or more RNA-recognition motif (RRM) at the N-terminus. In our investigation, one spot (no. 27) was identified as GRPs and this spot was down-regulated in the leaves of the stress rice plant. Zhu et al. (2013) reported that the expression of GRPs in *Pinellia ternata* leaves was slightly down-regulated at beginning, but after 12-h heat stress, the expression was significantly up-regulated. Three spots (nos. 25, 55, and 57) were identified as pathogen-related (PR) proteins and these spots were down-regulated in the leaves of the stress rice plant. It was also observed earlier that PR proteins are produced when tobacco leaves carrying N gene response to infection by TMV and form local lesions. However, at temperatures

Table 2 Functional characterization of protein spots up-regulated and down-regulated during high temperature in rice genotype (IET 21405): proteins in bold (1–4) were identified using MALDI-TOF analysis using MASCOT MS/MS ion search with significant threshold $P > 0.05$

Spot no.	Protein	pI	Molecular weight	Annotated species
Up-regulated proteins				
1	Ribulose biphosphate carboxylase small chain c	7.36	19,646	<i>Oryza sativa</i> Indica Group
2	Os03g078610	5.12	40,415	<i>Oryza sativa</i> Japonica Group
3	Alpha-galactosidase	6.82	46,191	<i>Oryza sativa</i> subsp. japonica
4	Chaperone protein ClpC3	5.27	10,104	<i>Oryza sativa</i> subsp. japonica
5	Peptidyl-prolyl cis-trans isomerase FKBP16-3	5.12	15,710	<i>Arabidopsis thaliana</i>
6	Actin-depolymerizing factor 3	4.90	16,176	<i>Oryza sativa</i> subsp. japonica (Rice)
7	Thiocyanate methyltransferase 1	4.61	27,408	<i>Arabidopsis thaliana</i>
8	Patatin-like protein 3	5.85	47,204	<i>Arabidopsis thaliana</i>
9	Ornithine decarboxylase	5.50	46,647	<i>Solanum lycopersicum</i> (tomato)
10	Magnesium transporter MRS2-A	5.45	46,294	<i>Oryza sativa</i>
11	Rho GTPase-activating protein 4	4.84	48,750	<i>Arabidopsis thaliana</i>
12	Photosystem II CP47	6.27	56,059	<i>Coffea arabica</i>
13	Chaperonin CPN60	6.21	58,782	<i>Brassica napus</i>
14	E3 ubiquitin-protein ligase	5.20	75,300	<i>Oryza sativa</i>
15	Protein NSP-interacting kinase	6.79	67,028	<i>Arabidopsis thaliana</i>
16	Putative boron transporter	6.88	76,736	<i>Arabidopsis thaliana</i>
17	Cellulose synthase	6.90	84,889	<i>Arabidopsis thaliana</i>
18	Syn-copalyl diphosphate synthase	5.60	87,465	<i>Oryza sativa</i>
19	Disease susceptibility protein	6.84	104,512	<i>Arabidopsis thaliana</i>
20	Protein suppressor of npr1-1	5.09	146,911	<i>Arabidopsis thaliana</i>
Downregulated proteins				
21	Monothiol glutaredoxin-S5	6.71	11,205	<i>Arabidopsis thaliana</i>
22	NAD(P)H-quinone oxidoreductase	6.54	11,262	<i>Nephrolepis olivacea</i>
23	Uncharacterized protein At1g24000	6.43	13,802	<i>Arabidopsis thaliana</i>
24	Mitochondrial import inner membrane translocase	5.83	10,840	<i>Oryza sativa</i> subsp. japonica (rice)
25	Pathogenesis-related protein	6.07	13,483	<i>Nicotiana tabacum</i> (common tobacco)
26	V-type proton ATPase subunit G1	5.77	12,397	<i>Arabidopsis thaliana</i>
27	Glycine-rich RNA binding	5.56	15,438	<i>Zea mays</i> (maize)
28	Probable phospholipid hydroperoxide glutathione peroxidase	5.72	18,596	<i>Citrus sinensis</i> (sweet orange)
29	ATP synthase epsilon chain	5.19	14,607	<i>Nicotiana tabacum</i>
30	Leucoagglutinating phytohemagglutinin	4.72	27,347	<i>Phaseolus vulgaris</i> (kidney bean)
31	Chitinase 2	5.83	32,583	<i>Oryza sativa</i> subsp. japonica
32	Ubiquitin receptor RAD23b	4.40	39,842	<i>Arabidopsis thaliana</i>
33	Putative pumilio homolog 17	6.00	38,511	<i>Arabidopsis thaliana</i>
34	BTB/POZ and MATH domain-containing protein 5	6.13	45,191	<i>Arabidopsis thaliana</i>
35	Chaperonin CPN60-2	5.34	57,570	<i>Cucurbita maxima</i>
36	Glucan endo-1,3-beta-glucosidase 12	5.22	57,303	<i>Arabidopsis thaliana</i>
37	Heat shock factor protein HSF8	5.17	57,701	<i>Solanum lycopersicum</i>
38	Chaperonin CPN60-1	5.09	57,398	<i>Cucurbita maxima</i>
39	Probable E3 ubiquitin-protein ligase	4.88	59,010	<i>Arabidopsis thaliana</i>
40	Pyruvate decarboxylase 1	5.69	66,213	<i>Arabidopsis thaliana</i>
41	Heat shock 70 kDa protein	5.42	66,984	<i>Solanum tuberosum</i>
42	RuBisCO large subunit-binding protein subunit alpha	5.58	61,863	<i>Chlamydomonas reinhardtii</i>
43	5-epiaristolochene synthase	5.33	65,099	<i>Capsicum annuum</i>
44	Heat shock 70 kDa protein	5.28	66,994	<i>Phaseolus vulgaris</i>
45	Heat shock 70 kDa protein 18	5.22	68,226	<i>Arabidopsis thaliana</i>

Table 2 continued

Spot no.	Protein	pI	Molecular weight	Annotated species
46	Heat shock 70 kDa protein	5.23	70,573	<i>Zea mays</i> (maize)
47	Probable mediator of RNA polymerase II	5.03	71,227	<i>Arabidopsis thaliana</i>
48	Heat shock 70 kDa protein	5.14	72,052	<i>Daucus carota</i>
49	Heat shock 70 kDa protein 3	4.98	71,017	<i>Arabidopsis thaliana</i>
50	Low temperature-induced 78 kDa protein	4.45	77,856	<i>Arabidopsis thaliana</i>
51	Ubiquitin carboxyl-terminal hydrolase	4.46	78,633	<i>Arabidopsis thaliana</i>
52	ATP synthase epsilon chain	6.62	14,984	<i>Acutodesmus obliquus</i>
53	17.4 kDa class I heat shock protein	5.20	17,440	<i>Arabidopsis thaliana</i>
54	Probable glutathione peroxidase	5.60	18,945	<i>Arabidopsis thaliana</i>
55	Pathogenesis-related protein	4.79	20,994	<i>Juniperus ashei</i>
56	22.3 kDa class VI heat shock protein	4.83	22,276	<i>Oryza sativa</i>
57	Pathogenesis-related protein 5	4.65	22,821	<i>Arabidopsis thaliana</i>
58	Histone chaperone ASF1B	4.01	24,701	<i>Arabidopsis thaliana</i>
59	Glutathione S-transferase	5.56	24,888	<i>Arabidopsis thaliana</i>
60	Patatin	5.25	40,009	<i>Solanum tuberosum</i>
61	Heat stress transcription factor	5.16	42,580	<i>Oryza sativa</i>
62	Heat stress transcription factor	5.34	46,245	<i>Arabidopsis thaliana</i>
63	Polygalacturonase	4.63	48,322	<i>Arabidopsis thaliana</i>
64	Protein BONZAI 1	5.67	62,989	<i>Arabidopsis thaliana</i>
65	Acyclic sesquiterpene synthase	5.77	67,350	<i>Zea mays</i>
66	Protein VERNALIZATION INSENSITIVE 3	5.88	69,348	<i>Arabidopsis thaliana</i>
67	Syn-copalyl diphosphate synthase	5.22	82,160	<i>Oryza sativa</i>
68	Probable disease resistance protein	5.53	105,062	<i>Arabidopsis thaliana</i>
69	Putative disease resistance protein	5.58	158,908	<i>Arabidopsis thaliana</i>
70	Indole-3-acetaldehyde oxidase	5.52	15,485	<i>Arabidopsis thaliana</i>
71	ARF guanine-nucleotide exchange factor GNL2	5.01	156,243	<i>Arabidopsis thaliana</i>
72	Dicer-like protein 4	6.30	191,280	<i>Arabidopsis thaliana</i>
73	Dicer-like protein 4	6.30	191,280	<i>Arabidopsis thaliana</i>

Fig. 3 Distribution pattern of the identified proteins (up-regulated and down-regulated) in rice genotype under high temperature, according to their biological function

higher than 30 °C, infected leaves carrying the N gene do not show necrotic lesions and TMV multiplies systemically in leaves with no PR proteins being induced under these conditions (Kaneko et al. 2016).

Ten percent proteins were differentially expressed in rice upon elevated temperature related to protein synthesis and transport. Heat shock protein, singly or in the form of chaperone is responsible for protein synthesis, targeting, maturation and degradation, and function in protein and membrane stabilization, and protein renaturation under heat damage condition (Kaneko et al. 2016). Variation in cellular concentration of aminoacyl tRNA synthetases, eight ribosomal proteins, EF-Tu had been observed, suggesting a severe affect of high temperature stress on protein biosynthesis (Li et al. 2013).

Elevated temperature, changes the abundance of proteins involved in energy pathways suggesting a close connection between these processes and heat stress. Plants required a large quantity of ATP for sufficient energy for growth, development, and stress responses. Among all, 2% proteins were involved in photosynthesis, tricarboxylic acid (TCA) cycle, glycolysis and other proteins for energy production. Previous studies recorded enhanced expression of protein related to the pyruvate dehydrogenase (PDH) enzyme complex (PDC), PDH E1 subunit, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and malate dehydrogenase (MDH) in rice leaves under heat stress (Han et al. 2009). It appears that the plant energy production systems are highly affected by elevated temperature, since many proteins involved in energy production were down-regulated. Proteins such as inorganic pyrophosphatase (PPase), Mt ATP synthase beta chain, and Ferredoxin-NADP(H) oxidoreductase (FNR) were decreased in abundance by heat treatment (Scafaro et al. 2010).

Among all the analyzed protein spots, 5% protein was found to be associated with plant metabolism and signal transduction. The protein quality control system may play an important role in the production of osmotic regulators. The improved metabolism of nitrogen, sugars and sugar alcohols may be an important tolerance mechanism induced by elevated temperature (Dutta et al. 2009).

MALDI-TOF-based identification of four selected protein spots revealed them as Ribulose biphosphate carboxylase small chain c (spot 1), Os03g078610 (spot 2), alpha-galactosidase (spot 3) and Chaperone protein ClpC3 (spot 4) (Fig. 1). Ribulose biphosphate carboxylase (Ru-bisco) small chain c (spot 1) is required to allow the rapid formation of the critical carbamate in the active site of Rubisco which cooperates the re-establishment of cellular homeostasis following heat stress (Natarajan and Kuehny 2008). Alpha galactosidases (α -Gals) (EC 3.2.1.22) are a widespread class of enzymes that liberate galactose from the non-reducing end of sugars. Plants synthesize α -

galactosides such as raffinose and stachyose as the major energy storage molecules in leaves, roots, and tubers. The physiological function of alkaline α -galactosidase is not clear, although the enzyme has been suggested to play an important role in hydrolysis of the raffinose family of oligosaccharides (RFOs) (Lee et al. 2009). ClpB-cyt/HSP100 protein acts as chaperone, mediating disaggregation of denatured proteins. HSP100 proteins belong to ClpB family. Clp ATPases maintain quality of cellular proteins by performing the function of molecular chaperones and energy dependent proteases. Plant ClpC proteins are Caseinolytic Protease, which are the homolog of the *Escherichia coli* ClpA proteins, have been noted in the chloroplast stroma of several plant species. ClpCs are considered to be highly conserved proteins among different species. ClpC have been seen to be associated with ClpP in the stroma in an ATP dependent manner (Singh et al. 2010).

Conclusion

The result of current study provide clear evidence that proteomic analysis of 73 differentially expressed heat shock proteins in rice leaves under elevated temperature are found functionally related to mainly defense followed by energy metabolism, protein synthesis and trafficking, signal transduction etc. This study also provides pragmatic opportunities to elucidate gene functions of these heat shock proteins for their functional analysis in rice. Further studies should be targeted towards characterization of these proteins at molecular level which may provide comprehensive picture of these proteins at cellular level under elevated temperature stress condition.

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