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World's Hottest Chilli Bhut Jolokia (*Capsicum assamicum*) Proteome Revealed: Comparative Proteomic Analysis of Differentially Expressed Proteins

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Abstract

With proteomic analysis including 2-DE, image analysis, and protein identification with MALDI-TOF/MS, an investigation aiming at a enhanced understanding of the whole fruit proteome and differentially expressed proteins and/or gene products was carried out with total fruit extracts from Bhut Jolokia (*Capsicum assamicum*) and less pungent *Capsicum frutescens*. A total of 107 dominant protein spots have been identified here using a 2-DE/MS technique. Among the identified proteins, 14 proteins exhibited qualitative difference with unique expression in Bhut Jolokia and 6 proteins showed quantitative differential expression alterations. Among the 6 differential proteins, one was down-regulated and 5 were up-regulated. Apart from the unique and differential proteins for which a cluster of orthologous groups (COG) could not be assigned (36.84%), most of the dominant unique and differentially expressed proteins were assigned to the COG of energy production and conversion (21.05%) and also carbohydrate transport and metabolism (21.05%). This differential protein expression was further confirmed for selected candidates by semi-quantitative RT PCR and quantitative real time PCR. This is the first proteomic description of world's hottest chilli 'Bhut Jolokia' and a detailed functional analysis of these proteins would provide further information regarding complex cellular processes and mechanism of pungency in this important source of Capsaicinoids.

Keywords: *Capsicum assamicum*; Hottest chilli; Proteome; Differential proteomics; 2-DE/MS

Introduction

The genus Capsicum possesses a unique character called 'Pungency', due to the presence of the phenolic alkaloid Capsaicinoids [1]. Pungency is a highly desirable attribute for many uses, especially for food, medicinal, and industrial purposes. The predominant capsaicinoids present in the Capsicum fruits are capsaicin and dihydrocapsaicin in the ratio ranging between 1:1 and 2:1 [2].Various well known biological functions of capsaicinoids include: neurophysiologic and biochemical effects; antimicrobial, antioxidant, and anti-inflammatory properties; and the protective effect against various malfunctions such as cancer, atherosclerosis, and obesity [3-5].

In Indian systems of medicine including Ayurveda, Siddha and Unani [6-8], the dietary spices form important ingredients for treating chronic and acute diseases. Further, fresh and dried fruits as well as extracted oleoresin of pungent peppers are of high demand as the most heavily and frequently consumed spices throughout the world. One of such pungent pepper is the fruit of 'Bhut Jolokia', a source of the highly pungent capsacinoids and of antioxidants. It is native to the north eastern part of India, and is well appreciated due to its extremely high pungency and unique aroma and had been acknowledged as the hottest chilli in the world (Guinness World Records, 2006). It is known by various names in different regions such as 'Borbih jolokia', 'Bhoot jolokia' or 'Bih jolokia' in Assam, 'Naga king chilli' in Nagaland, 'Omorok' in Manipur and 'Ghost pepper' by the western media. Conventionally, it has also been used by different ethnic communities of the north eastern India in treating various human ailments, to tone up body muscles after heavy workouts, and hot infusions are used for toothache and muscle pain [9]. Its refreshing aroma, palatability and medicinal properties have attracted attention for use in pickle preparation, flavoring curries and for home remedies of ailments like gastritis, arthritis and chronic indigestion problems.

Owing to its very high capsaicinoids content, Bhut Jolokia (*Capsicum assamicum*) has become a plant of scientific attention as capsaicin is a potential molecule for the development of a new generation of analgesic/anti-inflammatory medicines [10]. Capsaicin can inhibit a variety of cancer cells [11,12] and also been shown to possess anti-inflammatory and antioxidant activities [5,12]. In addition, capsaicin prevents adipogenesis and obesity by activation of TRPV1 channels [13] and by decreasing energy intake [14]; it inhibits serum triglyceride via stimulation of lipid mobilization [15]. Chilli may play an important role in the process of chemoprevention [16] as the antioxidative capacity of chilli is higher than ginger, garlic, mint and onion [17].

Although, the biosynthetic pathway of capsaicinoids was first outlined by Bennet and Kirby [18], many of the enzymes involved in

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capsaicin biosynthesis are not yet well characterized and the regulation of the pathway remains obscure. Recently, a single dominant gene *C*, present in chromosome 2 and responsible for pungency is identified, along with several markers that co-segregated with *Pun1* [19]. *AT3*, an acyltransferase, is known as a strong candidate gene product for *Pun1*, which resides at the *pun1* locus, and encodes a putative acyltransferase [20]. In non-pungent chillies, the recessive allele, *pun1*, is present in the homozygous condition. However, no information is available on the degree of capsaicinoids accumulation in the genus Capsicum by the action of specific genes. Earlier, for the study of differential metabolic processes in plants differential gene expression based on mRNA screenings were used. Transcriptomic studies are inherently associated with several limitations including lack of information pertaining to post-transcriptional modifications, proteolytic cleavage, etc. In this context, proteomics is an ultimate technology for detecting changes in gene expression at protein level, as it allows comparison of two or more samples at a reasonably higher level [21].

The present investigation was carried out with the objective of generating a reference proteome of the very pungent fruit of Bhut Jolokia (*Capsicum assamicum*) and investigating differences in its proteome profile in comparison with less pungent closely related *Capsicum frutescens*. This was accomplished through a proteomic approach based on two-dimensional gel electrophoresis (2-DE) followed by in-gel tryptic digestion and MALDI-TOF-MS/MS for protein identification. The identification of differential proteins in the present investigation advances our understanding of the reasons of pungency in the hottest chilli 'Bhut Jolokia', and opens up opportunity for interpreting the metabolic basis of acute hotness in Bhut Jolokia. In our recent report we have discussed and reported a unique 13 bp deletion in the 5.8S region of 'Bhut Jolokia' through isolation and sequencing of the Internal Transcribed Spacer (ITS) region, [22], which may be correlated to its extreme pungency. However, to the best of our knowledge, this is the first proteomic elucidation of the hottest chilli Bhut Jolokia (*Capsicum assamicum*).

Materials and Methods

Plant material

Two types of pungent chilli, one the hottest chilli BhutJolokia (*Capsicum assamicum*) and other one *Capsicum frutescens* (locally called 'Mem Jolokia') were collected from forest village market of Tezpur, Assam, India. In all cases, the people selling the chilli fruits (and hence, chilli seeds), claimed that they had been collected from cultivated local chilli populations for both the chilli species. In our research, the experimental materials (chilli fruits) were obtained from the cultivated chilli plants raised from these chilli seeds, in the experimental farm of Defence Research Laboratory, Tezpur, Assam, India, in the summer of 2010. The mature fruits of 'Bhut Jolokia' were subconical to conical in shape with orange to red colour with a rough and dented skin. The mature fruits of *C. frutescens* were elongated, slender having red-orange red colour. Fruits of 'Bhut Jolokia' have extreme pungency and *C. frutescens* have less pungency.

Protein extraction

Protein was extracted from chilli fruit samples as per the method reported by Damerval et al. [23] with some modifications. Briefly, fruits from 'Bhut Jolokia' and *Capsicum frutescens* were ground to a fine powder in liquid nitrogen using mortar and pestle. About 1 g (1 part) of this fine powder was mixed with 10 ml (10 parts) of precipitation solution containing 10% w/v TCA and 0.07% w/v 2-mercaptoethanol in acetone. The suspension was incubated at –20°C for 60 min with intermittent mixing (every 10 min) using a cyclomixer (Bangalore Genie, India). Precipitated material was collected by centrifugation (25,000×g, 4°C, 15 min). The pellet was washed twice with acetone containing 0.07% w/v 2-mercaptoethanol and the precipitate was air dried for 20 min. The pellet was stored at -80°C until use. Proteins were dissolved from the dried precipitate into lysis-buffer (8 M urea, 2% CHAPS) by repeated pipetting and by using a cyclomixer. Insoluble material was removed by centrifugation (20,000×g, 20°C, 20 min) and the supernatant was clarified by passing through a 0.22 µm syringe filter (Millipore, India).

Total protein concentration was determined according to the method of Bradford [24] using Quick Start Bradford Protein Assay kit (Bio-Rad, USA) as per manufacturer's instructions. The protein concentration was calculated using bovine serum albumin (BSA) as standard.

2-DE

In order to improve focusing, proteins samples were purified using 2D-cleanup kit (Bio-Rad) and the protein pellet was finally resuspended in sample rehydration buffer (8M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG buffer pH 3–10). The isoelectric focusing was performed using immobilized pH gradient (IPG) strips (Bio-Rad, USA). IPG strips with a pH range from 4 to 7 were used for all the experiments. For the first dimension 250 and 500 µg of protein samples in 150 and 300 µl of rehydration solution was used to rehydrate IPG strips of 7 cm and 17 cm, respectively. The IPG strips were rehydrated overnight and then the proteins were focused for 10,000 VHr at 20°C under mineral oil. After focusing, the strips were incubated for 10 min, in 1 ml (for 7 cm strip in mini gel) or 4 ml (for 17 cm strip in maxi gel) of equilibration buffer I (6 M urea, 30% w/v glycerol, 2% w/v SDS and 1% w/v DTT in 50 mMTris/HCl buffer, pH 8.8) followed by equilibration buffer II (6 M urea, 30% w/v glycerol, 2% w/v SDS and 4% w/v iodo-acetamide in 50 mMTris/HCl buffer, pH 8.8). After the equilibration steps the strips were transferred to 12% SDS-PAGE for the second dimension by the method of Blackshear [25]. Protein spots were visualized by staining with Coomassie Brilliant Blue G-250. Gel images were captured by GS800 densitometer (Bio-Rad, USA). Relative abundance of the spots and the differential protein expression were determined by PD Quest software (Bio-Rad, USA).Two independent experiments were carried out for each type of chilli, and replicate gels (n=4) were generated from each independent experiment for the differential study. Fruits from 4-5 plants were pooled together for each sample preparation.

Identification of protein spots by mass spectrometry

Protein spots were excised from maxi gel with the help of thin-walled PCR tubes (200 µl) appropriately cut at the bottom with the help of fresh surgical scalpel blade. Care was taken not to contaminate the spots from adjoining proteins or with skin keratin. Gel pieces excised from 2-DE gels were destained at room temperature with 200 µL 50% ACN/50 mM NH_4HCO_3 for 1 h. Gel pieces were dried and 100ng trypsin (Promega, USA) in 50mM NH_4HCO_3 was added to each piece. Tryptic digestion was carried out overnight at 37°C. Peptides were extracted with 60% acetonitrile and 0.1% trifluoro-acetic acid (TFA), dried, and resuspended in 0.5% TFA before MS analysis.

Excised and digested proteins were identified by Applied Biosystem 4800 plus MALDI TOF/TOF Analyzer (AB Sciex, USA) using conditions as previously described Kumar et al. [26]. Peptides after

digestion were mixed with equal volume of the CHCA matrix solution (10 mg/ml) and spotted onto the target plate. A default calibration was applied using a six component peptide standards in a mass range of 905-3660 Da, spotted onto 13 callibration points on 384-well MALDI plate. MS mass spectra were recoded in the reflector positive mode using a laser operated at a 200 Hz repetition rate with a wavelength of 355 nm. The accelerated voltage was operated at 2 kV. The MS/MS mass spectra were acquired by the data dependent acquisition method and 20 strongest precursors were selected between 850 and 4000 Da and filtered with a signal-to-noise ratio greater than 20 from one MS scan.

All MS and MS/MS spectra were obtained by accumulation of at least 1200 and 1600 laser shots, respectively. MS and MS/MS data were analyzed and peak list were generated using the 4000 Series Explorer Software v. 3.5 (Applied Biosystems). A peak intensity filter was used with no more than 50 peaks per 200 Da in the setting parameter of MASCOT search after acquisition. MS/MS peaks were selected based on a signal-to-noise ratio greater than 10 over a mass range of 60–20 Da below the precursor mass. MS and MS/MS data were analyzed using Protein Pilot version 2.0 (Applied Biosystem) employing the MASCOT 2.0 search engine (Matrix Science, London, UK). The peak list was searched against the taxonomy group Green Plants at non-redundant protein sequence database of NCBI with 1158709 sequence entries. Search parameters were as follows: trypsin digestion with one missed cleavage, variable modifications (oxidation of methionine and carbamidomethylation of cysteine), and the peptide mass tolerance of 50ppm for precursor ion and mass tolerance of ± 0.6 Da for fragment ion with +1 charge state. For all proteins successfully identified by MS/MS, MASCOT score greater than 62 was accepted as significant (p-value < 0.05). For the proteins studied, a match with significant score in the closely related genus *Solanum, Nicotiana* and species *Capsicum annum* and *Capsicum chinense* was obtained, using Mascot search engine. False Discovery Rate (FDR) is estimated in MASCOT by searching against a decoy database where Mascot generates a randomized sequence of the same length for every sequence in the target database.

For differential study, results from four replicate gels of Bhut Jolokia (*C. assamicum*) were computationally combined using PD Quest (Bio-Rad Laboratories, Hercules, CA) software and matched spots were compared with that of *C. frutescens*. Differentially expressed spots were manually curated for their consensus among replicates and to eliminate any possible artifacts. Spot intensities were normalized by total valid spot intensities and mean of values from duplicate analytical gels from four replicates (two biological with two analytical replicates each) were subjected to paired t-test analysis. Protein spots showing altered expression between Bhut Jolokia (*C. assamicum*) and *C. frutescens* (|ratio| ≥ 1.5, $p \leq 0.05$) were marked and excised.

Bioinformatic analysis

In all the cases, proteins were identified as homologues in the closely related genus and species. Homology searches were carried out using the BLAST and PSI-BLAST protein algorithm against the GenBank non-redundant protein database at http://www.ncbi.nlm.nih.gov. The theoretical molecular weights and isoelectric points were determined using the Compute pI/Mw algorithm at expasy server (http://ca.expasy.org). Cluster of orthologous group (COG) for the identified proteins was determined using COGNITOR program at http://www.ncbi.nlm.nih.gov/ COG/. The identified homologues in 'Bhut Jolokia' were searched for Pfam, a database (http://pfam.sanger.ac.uk/) having large collection of protein families, each represented by multiple sequence alignments and hidden Markov models [27]. All identified protein sequences were searched using the PlantPLoc program (http://www.csbio.sjtu.edu.cn/cgi-bin/PlantPLoc.cgi) to predict their subcellular localization [28]. Gene ontology for the identified proteins was predicted from http://www.uniprot.org.

Semiquantitative reverse transcription PCR

Total RNA was extracted from the fruits of each plant essentially as described by Choi et al. [29]. Each RT reaction was run in a total volume of 20 µL with 500 ng total RNA as template and supplied RT primer mix (Qiagen, India) as primers. For each reaction 25–30 amplification cycles were used to ensure linearity of response and the reaction product were visualized on 1.0% agarose gels. The PCR primers for each tested gene product are detailed in Table 1. All experiments were repeated at least three times.

Quantitative real time PCR

We determined the expression of selected genes of interest at the transcriptional level using quantitative real time PCR (qRT-PCR). An Unknown Protein (Spot No. 47) and LEXYL2 (Spot no. 90) were interesting because their protein levels showed significant up-regulation in Bhut Jolokia (*C. assamicum*) compared to that of *C. frutescens*. Quantitative real-time PCR was performed in triplicate for each sample with QuantiTect™ SYBR® Green PCR Kit (Qiagen, India) following manufacturer's instructions with primers listed in Table 1

Gene	Primer Name	Oligomer	Product Size (bp)	Temperature (°C)/time (sec)		
				Denaturation	Annealing	Elongation
Putative pathogenesis related protein	PR	Forward: TGAGTCCACAACCACA Reverse: GCGAGGAGGTACGCTTCGA	450	95/30	52/30	72/60
Pathogenesis-related protein 10	PR10	Forward: CCACAGCCTCAGTTGCCCCA Reverse:GGCGAGGAGGTATGCTTCGATGGC	432	95/30	57/30	72/60
NACA3	NACA3	Forward: CTCGCCGCCAAATTGGAAGA Reverse:CACGTTGCTGAGGTTGGGAGC	387	95/30	52/30	72/60
Triose phosphate isomerase cytosolic isoform-like	TPI	Forward: TGCAACCCCTGCACAAGCCC Reverse: GGCGCCTTTCTTCACCTCAGC	235	95/30	54/30	72/60
Unknown	Unkown	Forward: GGCCACTTTGCCCGTTCCAAT Reverse:CCATGGCAGGCACCGGCAAG	533	95/30	54/30	72/60
Enolase-like	ENOL	Forward: TCAAAATGAGTGGGGTTGGTGCAA Reverse: AACCACCCTCGTCACCGACA	353	95/30	54/30	72/60
LEXYL2	LEXYL2	Forward: CACGGCAGGTTCATTGCCTCT Reverse:TGCAGCTGCTACTTTCTTGGCA	229	95/30	52/30	72/60

Table 1: List of primers and optimal PCR amplification conditions used for the semiquantitative RT-PCR and quantitative Real-time RT-PCR.

under optimized amplification conditions. 18S rRNA gene was used as an internal control.

Results

Protein identification

Proteins from the fruits of Bhut Jolokia (*C. assamicum*) and *C. frutescens* were separated in the first dimension by a pH 4 to 7 immobilized pH gradient gel (length, 7cm and 17 cm) and then in the second dimension by a 12% polyacrylamide gel. For 2-DE analyses, two independent experiments were carried out for each type of chilli, and replicate gels (n=4) were generated from each independent experiment to allow statistical analysis for the differential study. Student's t-test was used to determine if the relative change in protein expression in 'Bhut Jolokia' and *Capsicum frutescens* was statistically significant. In 'Bhut Jolokia' a total of 107 dominant protein spots (Figure 1) detected on the 2-DE gels were identified. The spots are labelled on the gel according to the numbers presented in Table 2. Spots were excised, analyzed after in-gel digestion with trypsin using MALDI-TOF-MS/MS as shown in Table S1 in the supplemental material.

MALDI-TOF-MS analysis resulted in identification of 107 proteins spots in 'Bhut Jolokia' as homologues of proteins from three genera of the family Solanaceae viz. *Solanum, Nicotiana, Capsicum, Ricinus, Datura* and also from other related genera. Interestingly, some of the proteins were identified in more than one spot on the 2-D gels. The gels were analyzed quantitatively to determine the relative abundance of spots and also the fold difference of expression in 'Bhut Jolokia' specific proteins taking the most abundant spot as 100% value (Table 2, Supplementary figure S1).The comparative image analysis using PD Quest software revealed several spots which were unique proteins as well as proteins with upto 17 fold higher and 0.288 fold lower expression, which were also selected and identified using MALDI-MS/MS.

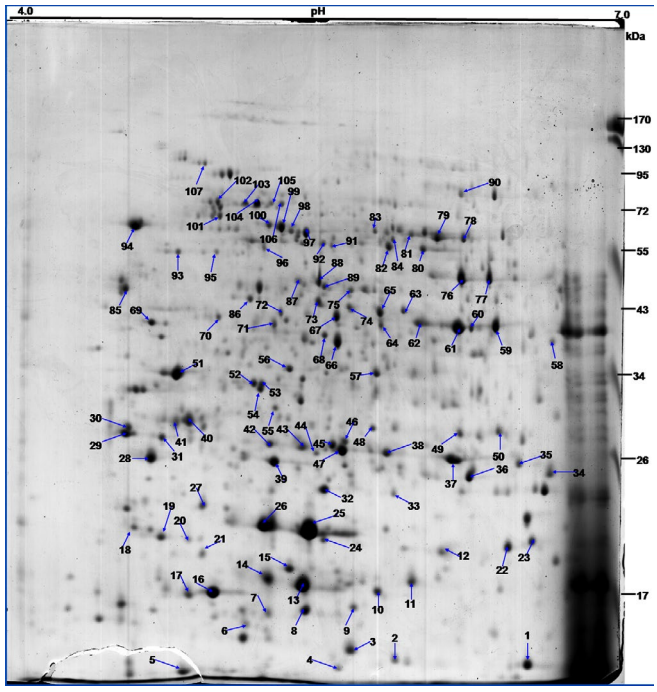

Figure 1: Total Spots numbered.

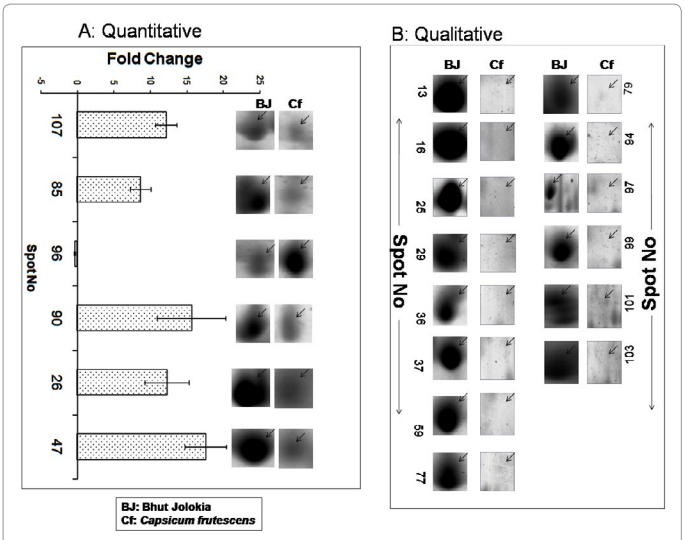

Figure 2: Fold change and Single Spot Component.

We estimated the MW and pI values of the protein spots on the 2-DE gels and compared them with theoretical MW and pI values of corresponding proteins from 'Bhut Jolokia'. Most of the experimental values matched well with theoretical values, indicating unambiguous identification (Table 2). Any discrepancies between experimental and theoretical masses might have been caused by post-translational proteolytic processing and modification. The differences between the two pI values might be attributed to the cleavage of alkaline regions and phosphorylation of multiple residues. Moreover, the homologues in other species could be of different primary sequence exhibiting altered mobility as the whole genome data for the two species is not available.

The proteome profiles from fruit tissue lysates of two kinds of peppers were tested and compared. The relative spot density analysis on the 2-DE gel and the identification data suggest a transposon protein (spot no. 16), belonging to a family of proteins of unknown function, was the most abundant unique protein present in 'Bhut Jolokia' as seen on 2-DE gel (Table 2). The other abundant proteins in the total protein extract of 'Bhut Jolokia' fruit (Table 2, Figure 1) included pathogenesis-related protein (spot no. 26), putative replication factor A (spot no. 40), fibrillin (spot no. 51), calreticulin (spot no. 94), putative pathogenesis related protein (spot no. 14), and unknown proteins (spot no. 5 and 47), Constitutive plastid-lipid associated protein (spot no. 3), Malate dehydrogenase (spot no. 61), Protein P21 (spot no. 34), Actin (spot no. 88), Enolase-like (spot no. 79), Eukaryotic translation initiation factor 5A-2 (spot no. 11), Chaperonin 10 (spot no. 39), Pathogenesis-related protein R major form (spot no. 28), Nucleoside diphosphate kinase (spot no. 1) and Thioredoxin peroxidase (spot no. 29) etc. The 2-DE gel pattern (Figure 1) also indicated that Constitutive plastid-lipid associated protein (spot no. 3, 7), Glycine-rich RNA-binding protein RGP-1c (spot no. 6, 8), Putative pathogenesis related protein (spot no. 13, 14), Pathogenesis-related protein 10 (spot no. 25, 26), Triose phosphate isomerase cytosolic isoform (spot no. 37, 38), Unknown (spot no. 43, 47), Malate dehydrogenase (spot no. 59, 60, 61, 62, 89), 3-oxoacyl-[acyl-carrier-protein] synthase (spot no. 76, 77), Enolase-like (spot no. 79, 81), Abscisic stress ripening protein (spot no. 103, 104) etc. existed as multiple electropherotypes.

There were often differences in their observed and theoretical molecular masses, (Table 2) which was more pronounced for those proteins which existed as multiple electropherotypes. Broadly,

Table 2: Protein spots identified from fruit lysate of 'Bhut Jolokia' along with differentially expressed proteins.

Spot No.	Protein Identity (Accession. No.)	Organism	M_r/pI		Fold Changes	Relative abundance[b]
			Theoretical[a]	Observed		
1	Nucleoside diphosphate kinase (gi|12230332)	*Capsicum annum*	16.3/6.3	13.0/6.6	–	13.47
2	Thioredoxin H-type 1 (gi|267124)	*Nicotiana tabacum*	13.9/5.6	13.0/5.9	–	8.12
3	Constitutive plastid-lipid associated protein (gi|75266239)	*Solanum lycopersicum*	19.7/8.8	15.0/5.7	–	16.02
4	Hypothetical protein (gi|302836253)	*Volvox carteri*	13.6/9.1	14.0/5.5	–	4.07
5	Predicted protein (gi|168009197)	*Physcomitrella patens*	14.1/4.6	12.0/4.9	–	18.47
6	Glycine-rich RNA-binding protein RGP-1c (gi|45533923)	*Nicotiana sylvestris*	13.2/9.0	15.0/5.2	–	5.21
7	Constitutive plastid-lipid associated protein (gi|75266239)	*Solanum lycopersicum*	19.7/8.8	18.0/5.3	–	6.35
8	Glycine-rich RNA-binding protein RGP-1c (gi|45533923)	*Nicotiana sylvestris*	13.2/9.0	18.0/5.5	–	11.78
9	Superoxide dismutase [Cu-Zn], chloroplastic (gi|134682)	*Solanum lycopersicum*	22.2/5.7	18.0/5.7	–	5.82
10	Unknown (gi|118482257)	*Populus trichocarpa*	15.2/5.4	20.0/5.9	–	2.27
11	Eukaryotic translation initiation factor 5A-2 (gi|20138707)	*Solanum lycopersicum*	17.5/5.7	21.0/6.0	–	13.92
12	DH putative beta-hydroxyacyl-ACP dehydratase (gi|193290688)	*Capsicum annuum*	23.9/9.3	23.0/6.2	–	9.54
13	Putative pathogenesis related protein (gi|58531054)	*Capsicum chinense*	17.1/5.2	22.0/5.5	∞↑	0.54
14	Putative pathogenesis related protein (gi|58531054)	*Capsicum chinense*	17.1/5.2	22.0/5.3	–	23.25
15	Putative Cu/Zn superoxide dismutase (gi|171854653)	*Capsicum chinense*	15.2/5.1	23.0/5.4	–	11.88
16	Transposon protein, putative, CACTA, En/Spm sub-class (gi|77553508)	*Oryza sativa Japonica*	91.0/6.4	19.0/5.0	∞↑	40.13
17	Unknown (gi|118481397)	*Populus trichocarpa*	23.0/7.6	19.0/4.9	–	12.31
18	P23 protein (gi|587546)	*Solanum tuberosum*	18.7/4.5	25.0/4.7	–	5.64
19	Copper chaperone (gi|15228869)	*Arabidopsis thaliana*	12.9/4.9	23.0/4.8	–	10.07
20	Conserved hypothetical protein (gi|255542318)	*Ricinus communis*	19.6/4.9	25.0/4.9	–	2.88
21	Pathogenesis-related protein 10 (gi|60542787)	*Capsicum chinense*	17.2/4.8	25.0/5.0	–	4.99
22	Thioredoxin peroxidase (gi|18654477)	*Capsicum annum*	17.3/6.0	24.0/6.5	–	13.62
23	Putative transcription factor Btf3 (gi|121551087)	*Capsicum annuum*	17.6/6.3	24.0/6.6	–	9.7
24	Phospholipid hydroperoxide glutathione peroxidase (gi|31872080)	*Solanum lycopersicum*	18.9/6.3	26.0/5.6	–	4.32
25	Pathogenesis-related protein 10 (gi|85700977)	*Capsicum baccatum*	17.2/5.2	25.0/5.5	∞↑	100
26	Pathogenesis-related protein 10 (gi|85700977)	*Capsicum baccatum*	17.2/5.2	25.0/5.3	12.34 ↑	38.40
27	ATP synthase subunit delta', mitochondrial (gi|2493046)	*Ipomoea batatas*	21.3/5.9	27.0/5.0	–	8.71
28	Pathogenesis-related protein R major form (gi|131015)	*Nicotiana tabacum*	24.6/5.3	29.0/4.7	–	13.84
29	NACA3 (gi|240256288)	*Arabidopsis thaliana*	22.0/4.4	31.0/4.6	∞↑	12.49
30	Acidic 27 kDa endochitinase (gi|544010)	*Solanum lycopersicum*	26.5/4.6	32.0/4.6	–	10.21
31	Unknown (gi|255641541)	*Glycine max*	25.9/4.7	31.0/4.8	–	7.16
32	23kDa polypeptide of the oxygen evolving complex of photosystem II (gi|146454486)	*Sonneratia alba*	25.1/5.9	27.0/5.6	–	8.57
33	Proteasome subunit beta type, putative (gi|255558626)	*Ricinus communis*	22.8/5.1	27.0/5.9	–	3.74
34	Protein P21 (gi|129320)	*Glycine max*	21.4/4.84	27.0/6.7	–	14.43
35	Proteasome subunit beta type-1 (gi|17380185)	*Petuniax hybrida*	24.6/6.3	28.0/6.5	–	7.87
36	Dehydroascorbate reductase (gi|160347100)	*Nicotiana tabacum*	23.4/6.0	28.0/6.3	∞↑	10.35
37	Triose phosphate isomerase cytosolic isoform (gi|38112662)	*Solanum chacoense*	27.0/5.7	29.0/6.2	–	6.96
38	Triose phosphate isomerase cytosolic isoform-like (gi|38112662)	*Solanum tuberosum*	27.0/5.7	30.0/5.9	∞↑	11.58
39	Chaperonin 10 (gi|3057150)	*Arabidopsis thaliana*	26.9/8.8	32.0/5.3	–	13.02
40	Replication factor A 1, rfa1, putative (gi|255546005)	*Ricinus communis*	75.3/8.1	33.0/4.9	–	32.11
41	Cysteine protease Cp1(gi|146215994)	*Actinidia deliciosa*	39.1/6.1	33.0/4.8	–	8.98
42	GSTL2 (gi|15233164)	*Arabidopsis thaliana*	33.0/6.7	30.0/5.3	–	7.85
43	Unknown (gi|255645535)	*Glycine max*	33 1/6.8	32.0/5.5	–	12.21
44	Chaperonin 21 precursor (gi|7331143)	*Solanum lycopersicum*	26.5/6.8	31.0/5.5	–	3.41
45	Unknown (gi|77416969)	*Solanum tuberosum*	25.6/5.3	30.0/5.6	–	12.70
46	Proteasome-like protein alpha subunit (gi|77999303)	*Solanum tuberosum*	27.1/5.6	30.0/5.7	–	10.66
47	Unknown (gi|255645535)	*Glycine max*	33.1/6.8	32.0/5.7	17.65↑	18.79
48	L-ascorbate peroxidase (gi|804973)	*Capsicum annuum*	27.3/5.3	33.0/5.8	–	4.82
49	Tropinone reductase 1 (gi|1717752)	*Datura stramonium*	29.5/6.1	32.0/6.3	–	6.97
50	Cytosolic ascorbate peroxidase (gi|62910196)	*Capsicum annuum*	27.4/5.7	31.0/6.5	–	7.85
51	Fibrillin (gi|460761)	*Capsicum annum*	35.2/5.0	37.0/4.8	–	23.51
52	Unknown (gi|255638262)	*Glycine max*	34.7/5.5	35.0/5.2	–	7.49

53	Chloroplast managanese stabilizing protein (gi|283049930)	*Capsicum annuum*	29.9/8.1	35.0/5.3	–	12.02
54	Predicted protein (gi|224103823)	*Populus trichocarpa*	33.5/6.8	35.0/5.3	–	3.84
55	Harpin binding protein 1 (gi|38679329)	*Solanum tuberosum*	30.0/8.3	34.0/5.3	–	3.49
56	Hypothetical protein VITISV_014475 (gi|147856362)	*Vitis vinifera*	30.7/5.0	37.0/5.4	–	6.41
57	Phenylcoumaran benzylic ether reductase (gi|213385143)	*Nicotiana tabacum*	33.9/5.9	37.0/5.9	–	8.82
58	Predicted protein (gi|224063293)	*Populus trichocarpa*	31.5/5.8	39.0/6.7	–	3.36
59	Malate dehydrogenase (gi|68299213)	*Capsicum chinense*	19.9/5.3	42.0/6.5	∞↑	14.18
60	Malate dehydrogenase (gi|68299213)	*Capsicum chinense*	19.9/5.3	41.0/6.3	–	5.69
61	Malate dehydrogenase (gi|68299213)	*Capsicum chinense*	19.9/5.3	42.0/6.3	–	36.07
62	Malate dehydrogenase (gi|68299213)	*Capsicum chinense*	19.9/5.3	41.0/6.0	–	12.37
63	Glutamine synthetase GS1 (gi|209529862)	*Solanum tuberosum*	38.5/5.2	44.0/6.0	–	4.49
64	Putative pyruvate dehydrogenase E1 beta subunit (gi|193290724)	*Capsicum annuum*	44.2/6.0	42 0/5.9	–	6.25
65	Caffeic acid 3-O-methyltransferase (gi|30315948)	*Capsicum annum*	39.4/5.6	46.0/5.9	–	11.31
66	Putative enoyl-acyl-carrier-protein reductase (gi|193290684)	*Capsicum annuum*	41.6/8.8	41.0/5.7	–	8.18
67	Alpha-galactosidase (gi|34765755)	*Petunia x hybrida*	31.3/4.8	43.0/5.7	–	6.18
68	DegP protease precursor (gi|2565436)	*Arabidopsis thaliana*	46.1/6.0	41.0/5.6	–	4.42
69	Putative stress related chitinase (gi|62719021)	*Nicotiana tabacum*	9.0/3.9	42.0/4.7	–	8.03
70	Fructokinase 3 (gi|38604456)	*Solanum lycopersicum*	41.4/5.5	42.0/5.1	–	3.78
71	Cysteine synthase, chloroplastic/ chromoplastic (gi|11131628)	*Solanum tuberosum*	41.0/5.4	42.0/5.3	–	5.58
72	Hypothetical protein SELMODRAFT_111224 (gi|302795987)	*Selaginella moellendorffii*	35.5/5.1	43.0/5.4	–	3.77
73	Predicted protein (gi|224053535)	*Populus trichocarpa*	38.5/5.8	45.0/5.6	–	8.48
74	Caffeic acid O-methyltransferase (gi|12003964)	*Capsicum annuum*	39.3/5.5	44.0/5.7	–	5.32
75	Glutamine synthetase GS58 (gi|40457328)	*Nicotiana attenuata*	47.4/6.7	47.0/5.7	–	4.35
76	3-oxoacyl-[acyl-carrier-protein] synthase (gi|3599489)	*Capsicum chinense*	52.4/8.0	47.0/6.2	–	11.65
77	3-oxoacyl-[acyl-carrier-protein] synthase (gi|3599489)	*Capsicum chinense*	52.4/8.0	48.0/6.4	∞↑	6.05
78	Leucine aminopeptidase 2, chloroplastic (gi|2492530)	*Solanum lycopersicum*	59.5/8.1	53.0/6.3	–	6.26
79	Enolase-like (gi|82623425)	*Solanum tuberosum*	48.0/7.5	56.0/6.2	∞ ↑	13.51
80	UTP--glucose-1-phosphate uridylyltransferase (gi|136739)	*Solanum tuberosum*	51.8/5.7	52.0/6.0	–	5.74
81	Enolase-like (gi|82623425)	*Solanum tuberosum*	48.0/7.5		–	5.20
82	UTP--glucose-1-phosphate uridylyltransferase (gi|136739)	*Solanum tuberosum*	51.8/5.7	51.0/5.9	–	9.42
83	Enolase (gi|119354)	*Solanum lycopersicum*	47.7/5.6	60.0/5.8	–	3.13
84	Endopolygalacturonase (gi|78482998)	*Capsicum annuum*	33.9/6.8	54.0/5.9	–	3.09
85	Ankyrin-repeat protein HBP1 (gi|13310811)	*Nicotiana tabacum*	37.2/4.4	46.0/4.6	8.7 ↑	10.14
86	Adenosine kinase isoform 1T (gi|51949796)	*Nicotiana tabacum*	37.4/5.1	45.0/5.2	–	5.26
87	Actin-51 (gi|3219772)	*Solanum lycopersicum*	37.1/5.2	47.0/5.4	–	3.81
88	Actin (gi|158529884)	*Glycyrrhiza uralensis*	41.5/5.3	46.0/5.6	–	13.74
89	Malate dehydrogenase (gi|56562183)	*Solanum lycopersicum*	48.4/6.2	45.0/5.6	–	4.19
90	LEXYL2 (gi|37359708)	*Solanum lycopersicum*	68.8/8.0	71.0/6.3	15.68 ↑	4.67
91	Putative branched-chain alpha-keto acid dehydrogenase E2 subuni (gi|193290668)	*Capsicum annuum*	55.0/6.7	51.0/5.6	–	2.09
92	Hypothetical protein OsI_31140 (gi|125563499)	*Oryza sativa Indica*	50.7/5.7	52.0/5.6	–	2.98
93	RAD23-like (gi|77745475)	*Solanum tuberosum*	40.6/4.7	50.0/4.9	–	3.42
94	Calreticulin (gi|11131769)	*Nicotiana plumbaginifolia*	47.4/4.4	57.0/4.7	∞ ↑	23.46
95	RAD23 protein (gi|5640111)	*Solanum lycopersicum*	41.4/4.6	50.0/4.9	–	1.80
96	Serine carboxypeptidase III (gi|148469859)	*Nicotiana tabacum*	56.4/4.9	51.0/5.3	0.288↓	5.70
97	ATP synthase subunit beta, mitochondrial (gi|114421)	*Nicotiana plumbaginifolia*	59.8/5.9	57.0/5.5	∞↑	9.43
98	ATP synthase subunit beta, mitochondrial (gi|114421)	*Nicotiana plumbaginifolia*	59.8/5.9	60.0/5.4	–	5.02
99	ATP synthase subunit beta, mitochondrial precursor (gi|162462751)	*Zea mays*	59.0/6.0	56.0/5.4	∞↑	5.91
100	ATP synthase subunit beta, mitochondrial precursor (gi|114421)	*Zea mays*	59.8/5.9	60.0/5.3	–	5.20
101	Protein disulfide isomerase-like protein (gi|49257109)	*Glycine max*	58.6/5.0	58.0/5.1	∞↑	4.25
102	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (gi|1351030)	*Brassica napus*	57.6/4.8	70.0/5.1	–	5.19
103	Abscisic stress ripening protein (gi|607905)	*Solanum chacoense*	29.0/4.9	67.0/5.2	∞ ↑	4.32
104	Abscisic stress ripening protein (gi|607905)	*Solanum chacoense*	29.0/4.9	67.0/5.3	–	11.14
105	Unknown (gi|116787373)	*Picea sitchensis*	65.5/5.6	59.0/5.4	–	4.21
106	RuBisCO large subunit-binding protein subunit beta, chloroplastic (gi|2506277)	*Pisum sativum*	62.9/5.8	70.0/5.3	–	2.93
107	Stromal 70 kDa heat shock-related protein, chloroplastic (gi|1708311)	*Spinacia oleracea*	64.8/4.8	90.0/5.0	12.28 ↑	1.93

[a] Theoretical values were obtained with the help of an online tool at http://expasy.org/sprot/.

[b] Relative to most abundant protein on the gel. Spot volume was estimated as average OD x mm^2 in replicate gels using spot density determination tool in PD Quest software.

seventeen protein spots having much lower p*I* value than the theoretical value were identified (Table 2, Figure 1).

Based on the blastp search results, most of the proteins identified in the present investigation appeared to be moderately conserved (showing 78 to 100% amino acid identity and 90 to 100% amino acid similarity) among the different species of the genus Capsicum and different genera of the family *Solanaceae* (Table S2). Interestingly, transposon protein, putative, CACTA, En/Spm sub-class, which is the most abundant protein, showed no significant similarity with any other protein in the database by use of blastp (spot no.16, Table S2).

Using the protein sequences of the closest homolog, sub-cellular location and biological function were inferred from protein databases. The maximum numbers of identified proteins (47.82%) were predictably located in the chloroplast, followed by those in the cytosol (27.17%), the nucleus (19.57%), the mitochondria (15.21%), vacuole (4.34%), cell wall (4.34%), peroxisomes (23.26%) and endoplasmic reticulum (3.26%) (Table 3, Figure 4A).

Leaving apart proteins for which a cluster of orthologous groups (COG) could not be assigned (29.34%), classification according to biological function determined that the identified proteins largely belonged to the category of proteins involved in carbohydrate transport and metabolism (38.04%), followed by those involved in cell envelope biogenesis/outer membrane (29.34%), and posttranslational modification/protein turnover (22.82%) (Table 3, Figure 4B).

Differential protein expression in the fruit of 'Bhut Jolokia'

To detect the proteins variably expressed, 2-DE patterns from 'Bhut Jolokia' and *Capsicum frutescens* were compared. Among the 107 identified proteins, 14 unique proteins and 6 differential proteins were revealed by comparative image analysis between 'Bhut Jolokia' and *Capsicum frutescens* using PD Quest software (Table 2, Supplementary figure S1). Among the 6 differentially expressed proteins, one was down-regulated (spot no. 96) and 5 were up-regulated (spot no. 26, 47, 85, 90, 107). Pathogenesis-related protein 10 (spot no. 26) was the most abundant of the 6 differentially expressed proteins. Similarly, an unknown protein (spot no. 47) showing 17.65 fold difference of expression in 'Bhut Jolokia' was the second most abundant differential protein.

Amongst the specific and differential proteins, majority had chloroplast localization (42.1), followed by cytosol (31.5%), mitochondria (15.78%), endoplasmic reticulum (15.78%), nucleus (10.52%), peroxisome and vacuole (5.26%). The only protein lower in abundance in Bhut Jolokia, serine carboxypeptidase III (spot no. 96), belonging to the family of serine carboxypeptidase, for which a COG couldn't be assigned, was localized in peroxisome and vacuole (Table 3). Apart from the unique and differential proteins for which a cluster of orthologous groups (COG) could not be assigned (36.84%), most of the differential proteins were largely assigned to the COG of energy production and conversion (21.05%), followed by carbohydrate transport and metabolism (21.05%), and posttranslational modification, protein turnover (15.78%) (Table 3).

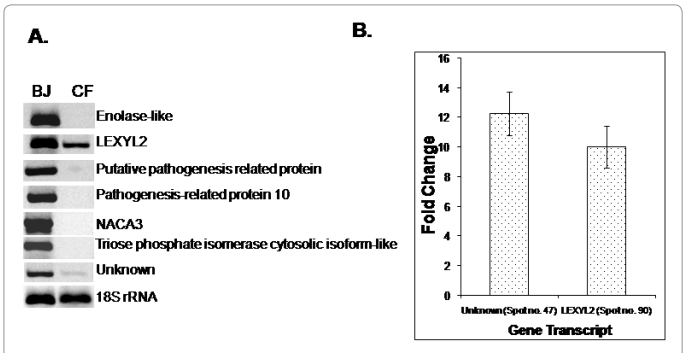

Figure 3: A) Semi-quantitative analysis of the RT-PCR results. **B)** 2-D image analysis and semi-quantitative RT-PCR.

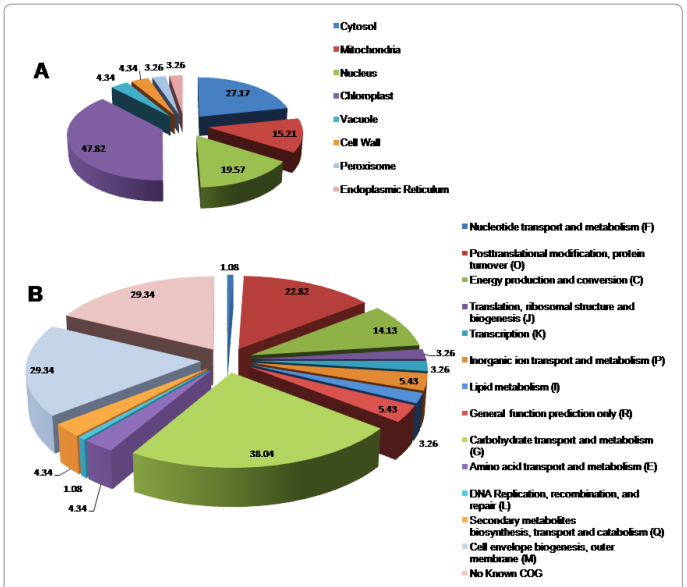

Figure 4: A) Maximum numbers of identified proteins, predictably located in the chloroplast. **B)** Cluster of orthologous groups.

The difference in protein expression pattern of Bhut Jolokia (*C. assamicum*) and *C. frutescens* was further verified at the transcript level. The five unique and two differential gene products were amplified by RT-PCR using total mRNA as the starting template. Overall results indicate that protein expression patterns observed in 2-DE gels are in linear correlation with significant difference of the corresponding mRNAs, which were tested using semi-quantitative analysis of the RT-PCR results (Figure 3A). As a more specific confirmation of the 2DE data, expression levels of an unknown protein (Spot no. 47) and LEXYL2 (Spot no 90) genes were tested using real-time PCR. The results demonstrate that the levels of transcripts for the two proteins were elevated in Bhut Jolokia (Figure 3B). These observations are parallel with the results obtained using 2-D image analysis and semi-quantitative RT-PCR (Figures 2A, 3A). Although the reason for the expression patterns of these gene products are not known at this moment, we hypothesize the possibility that these differential expressions may be related to the metabolism of the pungency in pepper. However, a more detailed study is required to investigate this prospect.

Discussion

It is of fundamental importance to understand the biological complexity in this special chilli 'Bhut Jolokia', which may be directly or indirectly correlated with its extremity of pungency making it the world's hottest chilli. Capsaicinoids are uniquely produced in the fruit of the genus Capsicum synthesized from phenylpropanoid intermediates and short-chain branched-fatty acids [18,30,31].

In Solanaceae, medium-length, branched-chain fatty acids are found as sugar esters in exudates from glandular trichomes that cover

Table 3: Proteins identified from 'Bhut Jolokia' fruit with functional category, Pfam, and gene ontology (Inferred from Electronic Annotation (IEA).

Spot No.	Protein Identity	Pfam match	Gene Ontology[a]	Localization[b]
Nucleotide transport and metabolism (F)				
1	Nucleoside diphosphate kinase (gi|12230332)	Nucleoside diphosphate kinase	Transcription, DNA-dependent, DNA binding, pyridoxal phosphate binding; sequence-specific DNA binding transcription factor activity	Cytosol, Mitochondria, Nucleus
Post translational modification, protein turnover (O) / Energy production and conversion (C)				
2	Thioredoxin H-type 1 (gi|267124)	Thioredoxin	Cell redox homeostasis, electron transport chain, glycerol ether metabolic process, electron carrier activity; protein disulfide oxidoreductase activity	Cytosol
101	Protein disulfide isomerase-like protein (gi|49257109)	Thioredoxin	Cell redox homeostasis, glycerol ether metabolic process, electron carrier activity; protein disulfide oxidoreductase activity	Endoplasmic reticulum
4	Hypothetical protein VOLCADRAFT_120809 (gi|302836253)	Thioredoxin	Cell redox homeostasis, glycerol ether metabolic process, electron carrier activity; protein disulfide oxidoreductase activity	Chloroplast
Translation, ribosomal structure and biogenesis (J)				
3, 7	Constitutive plastid-lipid associated protein (gi|75266239)	Endoribonuclease L-PSP	No related GO	Chloroplast
10	Unknown (gi|118482257)	Ribosomal protein family	No related GO	Nucleus
11	Eukaryotic translation initiation factor 5A-2 (gi|20138707)	Eukaryotic elongation factor 5A hypusine, DNA-binding OB fold	Peptidyl-lysine modification to hypusine, positive regulation of translational elongation, ribosome binding, translation elongation factor activity, translation initiation factor activity	Chloroplast
Transcription (K)				
6, 8	Glycine-rich RNA-binding protein RGP-1c (gi|45533923)	RNA recognition motif	Nucleic acid binding, nucleotide binding	Chloroplast
29	NACA3 (gi|240256288)	NAC domain	Protein transport, response to salt stress	Nucleus
87	Actin-51 (gi|3219772)	Actin	ATP binding	Cytosol
Inorganic ion transport and metabolism (P)				
9	Superoxide dismutase [Cu-Zn], chloroplastic (gi|134682)	Copper/zinc superoxide dismutase (SODC)	Superoxide metabolic process, metal ion binding, superoxide dismutase activity.	Chloroplast, Cytosol, Mitochondria
15	Putative Cu/Zn superoxide dismutase (gi|171854653)	Copper/zinc superoxide dismutase (SODC)	Superoxide metabolic process, metal ion binding, superoxide dismutase activity	Chloroplast
19	Copper chaperone (gi|15228869)	Heavy-metal-associated domain	Intracellular copper ion transport, oxidation-reduction process, superoxide dismutase copper chaperone activity, zinc ion binding	Cytosol, Mitochondria, Nucleus
48	L-ascorbate peroxidase (gi|804973)	Peroxidase	Embryo development ending in seed dormancy, hydrogen peroxide catabolic process, response to cadmium ion, heat / salt stress, L-ascorbate peroxidase activity, heme binding, metal ion binding	Peroxisome
50	Cytosolic ascorbate peroxidase (gi|62910196)	Peroxidase	Response to oxidative stress, heme binding, peroxidase activity	Peroxisome
Lipid metabolism (I)				
12	DH putative beta-hydroxyacyl-ACP dehydratase (gi|193290688)	FabA-like domain	Fatty acid biosynthetic process, lipid A biosynthetic process, hydro-lyase activity.	Chloroplast
Posttranslational modification, protein turnover, chaperones (O)				
17	Unknown (gi|118481397)	Redoxin	No related GO	Chloroplast
22	Thioredoxin peroxidase (gi|18654477)	Redoxin	Response to cadmium ion, peroxidase activity, peroxiredoxin activity	Cytosol
24	Phospholipid hydroperoxide glutathione peroxidase (gi|31872080)	Glutathione peroxidase	Response to oxidative stress, glutathione peroxidase activity, phospholipid-hydroperoxide glutathione peroxidase activity	Chloroplast, Mitochondria
31	Unknown (gi|255641541)	Proteasome subunit A	No related GO	Nucleus
33	Proteasome subunit beta type, putative (gi|255558626)	Proteasome subunit	Proteolysis involved in cellular protein catabolic process, threonine-type endopeptidase activity	Nucleus
35	Proteasome subunit beta type-1 (gi|17380185)	Proteasome subunit	Proteolysis involved in cellular protein catabolic process, regulation of plant-type hypersensitive response, response to salt stress, response to zinc ion, peptidase activity; threonine-type endopeptidase activity	Nucleus
36	Dehydroascorbate reductase (gi|160347100)	Glutathione S-transferase,	Response to oxidative stress	Cytosol
39	Chaperonin 10 (gi|3057150)	Chaperonin 10 Kd subunit	Protein folding; response to heat, ATP binding, copper ion binding	Chloroplast
42	GSTL2 (gi|15233164)	Glutathione S-transferase,	Protein glutathionylation, response to toxin, glutathione transferase activity.	--
44	Chaperonin 21 precursor (gi|7331143)	Chaperonin 10 Kd subunit	Protein folding, ATP binding	Chloroplast

45	Unknown (gi|77416969)	Proteasome subunit A N-terminal signature	Ubiquitin-dependent protein catabolic process, threonine-type endopeptidase activity	Nucleus
46	Proteasome-like protein alpha subunit (gi|77999303)	Proteasome subunit A N-terminal signature	Ubiquitin-dependent protein catabolic process, threonine-type endopeptidase activity	Cytosol, Nucleus
56	Hypothetical protein VITISV_014475 (gi|147856362)	Proteasome subunit A N-terminal signature	Response to arsenic-containing substance, ubiquitin-dependent protein catabolic process, threonine-type endopeptidase activity.	Nucleus
68	DegP protease precursor (gi|2565436)	Trypsin-like peptidase domain	Photosystem II repair, protein catabolic process, proteolysis, response to stress, serine-type endopeptidase activity	Chloroplast
102	RuBisCO large subunit-binding protein (gi|1351030)	TCP-1/cpn60 chaperonin family	Protein refolding, ATP binding	Chloroplast
105	Unknown (gi|116787373)	TCP-1/cpn60 chaperonin family	Protein refolding, ATP binding	Chloroplast
106	RuBisCO large subunit-binding protein (gi|2506277)	TCP-1/cpn60 chaperonin family	Protein refolding, ATP binding	Chloroplast
107	Stromal 70 kDa heat shock-related protein (gi|1708311)	Hsp70 protein	Protein folding, response to stress, ATP binding.	Chloroplast
Energy production and conversion (C)				
27	ATP synthase subunit delta' (gi|2493046)	ATP synthase, Delta/Epsilon chain	ATP synthesis coupled proton transport, hydrogen ion transporting ATP synthase activity, rotational mechanism, proton-transporting ATPase activity, rotational mechanism	Mitochondria
97 to 100	ATP synthase subunit beta, (gi|114421)	ATP synthase F1 beta subunit	ATP hydrolysis coupled proton transport, ATP synthesis coupled proton transport, ATP binding; hydrogen ion transporting ATP synthase activity, rotational mechanism, hydrogen-exporting ATPase activity, phosphorylative mechanism	Chloroplast, Mitochondria
54	Predicted protein (gi|224103823)	Inorganic pyrophosphatase	Defense response to bacterium, phosphate-containing compound metabolic process, inorganic diphosphatase activity, magnesium ion binding	Chloroplast, Cytosol
59 to 62	Malate dehydrogenase (gi|68299213)	Lactate/malate dehydrogenase	Carbohydrate metabolic process, malate metabolic process, L-malate dehydrogenase activity	Chloroplast
64	Putative pyruvate dehydrogenase E1 beta subunit (gi|193290724)	Transketolase, pyrimidine binding domain	Defense response to bacterium, catalytic activity	Chloroplast
72	Hypothetical protein (gi|302795987)	Transketolase, pyrimidine binding domain	Defense response to bacterium, catalytic activity	Mitochondria
73	Predicted protein (gi|224053535)	Transketolase, pyrimidine binding domain	Defense response to bacterium, catalytic activity	Mitochondria
89	Malate dehydrogenase (gi|56562183)	lactate/malate dehydrogenase, NAD binding domain	Carbohydrate metabolic process, malate metabolic process, malate dehydrogenase (NADP+) activity; nucleotide binding.	Chloroplast
91	Putative branched-chain alpha-keto acid dehydrogenase (gi|193290668)	Biotin-requiring enzyme	Fatty-acyl-CoA biosynthetic process, dihydrolipoyllysine (2-methylpropanoyl) transferase activity	Mitochondria
General function prediction only (R)				
30	Acidic 27 kDa endochitinase (gi|544010)	Chitinase class I	Cell wall macromolecule catabolic process, chitin catabolic process, defense response, chitinase activity	Vacuole
85	Ankyrin-repeat protein HBP1 (gi|13310811)	Ankyrin repeats (3 copies)	No related GO	Endoplasmic reticulum
Carbohydrate transport and metabolism (G)				
37, 38	Triose phosphate isomerase cytosolic isoform (gi|38112662)	Triosephosphate isomerase	Glycolysis, triose-phosphate isomerase activity	Chloroplast
43, 47	Unknown (gi|255645535)	Triosephosphate isomerase	Glycolysis, triose-phosphate isomerase activity	Chloroplast
79, 81	Enolase-like (gi|82623425)	Enolase, N-terminal domain	Glycolysis, magnesium ion binding, phosphopyruvate hydratase activity	Cytosol
70	Fructokinase 3 (gi|38604456)	pfkB family carbohydrate kinase	D-ribose metabolic process, ribokinase activity	Chloroplast
83	Enolase (gi|119354)	Enolase, N-terminal domain	Glycolysis, phosphopyruvate hydratase complex, magnesium ion binding; phosphopyruvate hydratase activity	Cytosol
86	Adenosine kinase isoform 1T (gi|51949796)	pfkB family carbohydrate kinase	AMP biosynthetic process, purine ribonucleoside salvage, adenosine kinase activity, phosphotransferase activity, alcohol group as acceptor.\	Chloroplast, Cytosol, Mitochondria
90	LEXYL2 (gi|37359708)	Glycosyl hydrolase family 3	Carbohydrate metabolic process, hydrolase activity, hydrolyzing O-glycosyl compounds	Cytosol, Mitochondria

92	Hypothetical protein OsI_31140 (gi|125563499)	Enolase, N-terminal domain	Glycolysis, trichome morphogenesis, magnesium ion binding; phosphopyruvate hydratase activity	Cytosol, Nucleus
Amino acid transport and metabolism (E)				
63	Glutamine synthetase GS1 (gi|209529862)	Glutamine synthetase, beta-Grasp domain	Glutamine biosynthetic process, nitrogen compound metabolic process, ATP binding; glutamate-ammonia ligase activity	Chloroplast, Cytosol
71	Cysteine synthase, chloroplastic / chromoplastic (gi|11131628)	Pyridoxal-phosphate dependent enzyme	Cysteine biosynthetic process from serine, cysteine synthase activity, transferase activity	Chloroplast, Mitochondria
75	Glutamine synthetase GS58 (gi|40457328)	Glutamine synthetase, beta-Grasp domain	Glutamine biosynthetic process; nitrogen compound metabolic process, ATP binding; glutamate-ammonia ligase activity	Chloroplast, Mitochondria
78	Leucine aminopeptidase 2, chloroplastic (gi|2492530)	Cytosol aminopeptidase family	Proteolysis, aminopeptidase activity, manganese ion binding, metalloexopeptidase activity	Chloroplast, Cytosol
DNA Replication, recombination, and repair (L)				
40	Replication factor A 1, rfa1, putative (gi|255546005)	Replication factor-A protein 1	DNA replication, DNA binding	Cell wall, Chloroplast, Cytosol, Nucleus
Secondary metabolites biosynthesis, transport and catabolism (Q)/ General function prediction only (R)				
41	Cysteine protease Cp1(gi|146215994)	Cathepsin propeptide inhibitor domain (I29)	Proteolysis, cysteine-type peptidase activity	Vacuole
49	Tropinone reductase 1 (gi|1717752)	short chain dehydrogenase	Tropane alkaloid biosynthetic process, nucleotide binding, tropine dehydrogenase activity	Chloroplast
74	Caffeic acid O-methyltransferase (gi|12003964)	Dimerisation domain	Lignin biosynthetic process, caffeate O-methyltransferase activity	Chloroplast
Lipid metabolism (I)/ Secondary metabolites biosynthesis, transport and catabolism (Q)				
76, 77	3-oxoacyl-[acyl-carrier-protein] synthase (gi|3599489)	Beta-ketoacyl synthase, N-terminal domain	Fatty acid biosynthetic process, 3-oxoacyl-[acyl-carrier-protein] synthase activity	Chloroplast
Cell envelope biogenesis, outer membrane (M)/ Carbohydrate transport and metabolism (G)				
57	Phenylcoumaran benzylic ether reductase (gi|213385143)	NmrA-like family	Nucleotide binding.	Cytosol
No known COG				
16	Transposon protein, putative, CACTA, En/Spm sub-class (gi|77553508)	No pfam	No related GO	Chloroplast
18	P23 protein (gi|587546)	Translationally controlled tumour protein	No related GO	Cytosol
20	Conserved hypothetical protein (gi|255542318)	PITH domain	Cell redox homeostasis, glycerol ether metabolic process, electron carrier activity; protein disulfide oxidoreductase activity	Chloroplast, Cytosol, Nucleus
21	Pathogenesis-related protein 10 (gi|60542787)	Pathogenesis-related protein Bet v I family	Defense response, response to biotic stimulus	Cytosol
23	Putative transcription factor Btf3 (gi|121551087)	NAC domain	No related GO	Nucleus
25, 26	Pathogenesis-related protein 10 (gi|85700977)	Pathogenesis-related protein Bet v I family	Defense response, response to biotic stimulus	Cytosol
103, 104	Abscisic stress ripening protein (gi|607905)	ABA/WDS induced protein	Response to stress	Nucleus
13, 14	Putative pathogenesis related protein (gi|58531054)	Pathogenesis-related protein Bet v I family	Defense response, response to biotic stimulus	Cytosol
5	Predicted protein (gi|168009197)	Profilin	Actin cytoskeleton organization	Chloroplast, Cytosol
32	23kDa polypeptide of O_2 evolving complex of photosystem II (gi|146454486)	PsbP	Photosynthesis, calcium ion binding	Chloroplast
34	Protein P21 (gi|129320)	Thaumatin family	No related GO	Vacuole
28	Pathogenesis-related protein R major form (gi|131015)	Thaumatin family	Defense response, response to biotic stimulus	Mitochondria
51	Fibrillin (gi|460761)	PAP_fibrillin	Structural molecule activity	Chloroplast
52	Unknown (gi|255638262)	Manganese-stabilising protein / photosystem II polypeptide	Photosynthesis, oxygen evolving complex, calcium ion binding	Chloroplast

53	Chloroplast managanese stabilizing protein (gi|283049930)	Manganese-stabilising protein / photosystem II polypeptide	Photosynthesis, photosystem II stabilization, oxygen evolving complex, calcium ion binding	Chloroplast
55	Harpin binding protein 1 (gi|38679329)	PAP_fibrillin	Chloroplast structural molecule activity	Chloroplast
58	Predicted protein (gi|224063293)	GRAM domain	No related GO	Cell wall, Nucleus
65	Caffeic acid 3-O-methyltransferase (gi|30315948)	Dimerisation domain	Lignin biosynthetic process, caffeate O-methyltransferase activity	Chloroplast
67	Alpha-galactosidase (gi|34765755)	Melibiase	Carbohydrate metabolic process, cation binding; raffinose alpha-galactosidase activity	Cell wall
69	Putative stress related chitinase (gi|62719021)	Podoplanin	No related GO	Cell wall
80, 82	UTP--glucose-1-phosphate uridylyltransferase (gi|136739)	UTP--glucose-1-phosphate uridylyltransferase	UTP:glucose-1-phosphate uridylyltransferase activity	Cytosol
84	Endopolygalacturonase (gi|78482998)	Glycosyl hydrolases family 28	Carbohydrate metabolic process, cellular cell wall organization, polygalacturonase activity	Cell membrane
88	Actin (gi|158529884)	Actin	Cytoskeleton, ATP binding.	Cytosol
93	RAD23-like (gi|77745475)	Ubiquitin family	Nucleotide-excision repair, proteasomal ubiquitin-dependent protein catabolic process, damaged DNA binding	Nucleus
94	Calreticulin (gi|11131769)	Calreticulin family	Protein folding, calcium ion binding	Endoplasmic reticulum
95	RAD23 protein (gi|5640111)	Ubiquitin family	Nucleotide-excision repair, proteasomal ubiquitin-dependent protein catabolic process, damaged DNA binding	Nucleus
96	Serine carboxypeptidase III (gi|148469859)	Serine carboxypeptidase	Proteolysis, serine-type carboxypeptidase activity	Peroxisome, Vacuole

[a]COGs were assigned after COGnitor search and functional role categories were assigned as per the descriptions in COG page at http://www.ncbi.nlm.nih.gov/COG.

[b]PlantPLoc: Predicting plant protein subcellular location (http://www.csbio.sjtu.edu.cn/cgi-bin/PlantPLoc.cgi).

the aerial epidermis [32-35]. In Capsicum, glandular regions in the fruit accumulate branched fatty acids as capsaicinoids [36,37].

Inspite of the first study of capsaicinoids biosynthetic pathway before three decades [18] many of the enzymes involved in capsaicin biosynthesis are yet to be characterized and the regulation of the pathway, subcellular localization etc. remains elusive. Capsaicinoid synthase has been implicated for the pungency of Capsicum fruits and is responsible for condensation between 8-methyl-nonenoic acid and vanillylamine to produce capsaicin; it has been shown that 8-methyl-nonenoic acid is the limiting factor in capsaicin synthesis determining the efficacy of capsaicin biosynthesis [38]. Assays for its activity using CoA-activated fatty acids and free fatty acids, had lead to the interpretation of a two step reaction by the formation of the acyl-CoA followed by transfer to vanillylamine [39]. Even after several studies, doubts exist with regard to putative capsaicin synthase enzyme itself. A candidate gene, *AT3*, encoding capsaicinoid synthase was believed to be critical in the evolution of pungency and appeared to be a hot spot for loss-of-pungency mutations [37]. In addition, a single dominant gene *C*, located in chromosome 2, is identified as responsible for pungency [19]. *AT3*, an acyltransferase, is identified as a gene product for *Pun1* [20]. In non-pungent Chillies, the recessive allele, *pun1*, is present in homozygous condition.

Differential patterns of gene product accumulation in the phenylpropanoid pathway were correlated with fruit pungency [40]. 3-oxoacyl-ACP synthase (KAS) expression was shown to be directly correlated with the level of capsaicin production and 8-methyl-nonenoic acid pool found to play a crucial role in determining the efficacy of capsaicin levels [41]. KAS accumulates in the placenta of pungent chilli fruits and accumulates in the epidermal cell layers of the placenta [42]. It was also shown that expression of putative aminotransferase (pAmt) is placenta-specific [40]. Using proteomic approach it was shown that KAS and pAmt are differentially up-regulated and transcripts are also differentially accumulated in pungent chillies that well correlated with the levels of vanillylamine and 8-methyl-nonenoic acid [21,39,41].

There is lots of debate regarding location and synthesis of capsaicinoids from vanillylamine and fatty acids [37]. One line of thought is that the site of capsaicinoid accumulation is the pepper seeds whereas other proposes that the placental dissepiments are the site of this accumulation and biosynthesis [36,37,43]. Also, it is proposed that once out of the cell, capsaicinoids accumulate underneath the cuticle in fluid-filled "blisters" [37], and they are near or in contact with the seeds.

The 2-DE coupled with MS is the method of choice for assessment of protein expression changes as it allows comparison of two or more samples at a reasonably higher level. High resolution 2-DE is already successfully used for studying complex patterns of protein expression in higher plants [30] and for monitoring global molecular responses following physiological responses. In this study, we used 'Bhut Jolokia' which is the hottest chilli having a heat content of 1,001,304 SHU [44] and *Capsicum frutescens* which is having medium pungency of around 276,500 SHU [45] as the starting materials for comparing the proteome profiles with the purpose of identifying the proteins specifically and differentially expressed in 'Bhut Jolokia'. Exploring the proteome differences in 'Bhut Jolokia' when compared with *Capsicum frutescens* provides a basis for the biochemical and physiological differences between these two chillies.

In the present investigation, the combination of 2-DE and MS has clearly identified major proteins in 'Bhut Jolokia' along with qualitative and quantitative differences in the protein expression pattern as compared to *Capsicum frutescens*. Accordingly, in 'Bhut Jolokia' a total of 107 dominant protein spots (Figure 1) detected on the 2-D gels were identified including 14 proteins specific to 'Bhut Jolokia'. And out of 6 differential proteins, 5 proteins showed over expression, while one abundant protein spot was down-regulated in whole fruit lysate of 'Bhut Jolokia' fruit as compared to *Capsicum frutescens* (Table 2, Figure

2 A & B). There are only a few reports on proteomic elucidation of Capsicum [42,46-48] and a similar study in the two species reported here is altogether lacking.

The study of the whole proteome of 'Bhut Jolokia' revealed that several of the identified proteins belonged to the functional categories of plant defence mechanisms and modulation of reactive oxygen species (ROS). For instance, Cu/Zn SOD (spot nos. 9, 15) is known to be involved in signalling pathways in plant defence mechanism, for example, the production of ROS and nitric oxide (NO), activation of mitogen-activated protein kinase (MAPK) and changes in defensive gene expression. The accumulation of ROS is one part of the signalling pathway involving plant defence mechanisms [49]. SOD, the metallo enzyme is also involved in the ROS detoxification and protects plant cells from the effect of ROS through catalysing the conversion of O_2^- to a signal molecule H_2O_2 [50]. Similarly, another protein found in this chilli is cytosolic ascorbate peroxidase (spot no. 50), which is a hydrogen peroxide scavenging enzyme whose supposed function is to protect the cell from hydrogen peroxide accumulation, particularly under stress conditions. It catalyzes the reduction of hydrogen peroxide using ascorbate as an electron donor, to yield water and oxidized ascorbate [51].

Also, many proteins identified as part of total proteome in this chilli are known to perform many important functions. These include, protein having roles in synthesis of nucleoside triphosphates (spot no. 1), chromoplastogenesis and stress (spot no. 3, 7), proteins involved in the initiation phase of eukaryotic translation (spot no. 11, 38), defense against chitin containing fungal pathogens (spot no. 30), photosynthesis and calcium ion binding (spot no. 32), regulator of cell cycle progression at G_1 (spot no. 34), glutathione transferase activity (spot no. 42) etc. One identified protein adenosine kinase isoform 1T (spot no. 118) in this study is known to play a key metabolic role in the recover synthesis of adenylates and methyl recycling and may also contribute to cytokinin inter-conversion [52].

Moreover, our study showed that 14 proteins exhibited qualitative difference and 6 proteins showed quantitative alterations. Among the 6 differential proteins, one was down-regulated and 5 were up-regulated. Dehydroascorbate reductase (spot no. 36) is a specifically expressed protein in 'Bhut Jolokia', known to regulate the cellular ascorbic acid redox state, which in turn affects cell responsiveness and tolerance to environmental ROS. DHAR affects the level of foliar ROS and photosynthetic activity during leaf development and as a consequence, influences the rate of plant growth and leaf aging [53]. Protein disulfide isomerase-like protein (spot no.101) of the family thioredoxin, is another specific protein identified in 'Bhut Jolokia'. Thioredoxins (Trxs) are small, multifunctional proteins with oxido-reductase activity and are ubiquitous in nature [54] and are reported to play protective role in the oxidative stress response. These are now known to be involved in a large panel of reactions related to metabolism, defense, and development [55]. Out of the six protein spots (spot nos. 13, 14, 21, 25, 26, 28) which were identified as pathogenesis-related proteins in 'Bhut Jolokia', two spots (spot nos. 13, 25) were specifically and one spot (spot no 26) was differentially expressed. It is known that attack of plants by pathogens such as fungi, bacteria, and viruses induce the expression of "pathogenesis-related proteins" (PRs) and these play a general role in plant's defense systems. PRs of class 10 are found as abundant in higher plants having a molecular weight of about 17 kDa and are found in the cytosol. Also, reports are there that some of these proteins are induced under stress conditions as part of the plant defense mechanism [56].

Except for the putative antiviral function of the PR-10 RNase [57], involvement of PR proteins on plant virus resistance is not known [58]. Hence, as has been described for certain PR proteins, their role is thought to be in improved host resistance against other plant pathogens, such as fungi and bacteria [58,59]. Also, PR-proteins are found to have a role in the mobilization of nutrients from the virus-damaged tissues, or in the protection against viral cellular injury [60]. Additionally, tobacco plants senescence study indicated that the accumulation of PR transcripts to be related to the mechanism of senescence and cellular damage [58,61].

Four protein spots (spot nos. 97, 98, 99, 100) were identified as ATP synthase subunit beta, mitochondrial, of which two spots (spot nos. 97, 99) are specifically expressed in 'Bhut Jolokia'. ATP synthase is known to involve in photosynthesis [62] suggesting that 'Bhut Jolokia' plants may require the energy to promote enzyme activities towards synthesis of capsaicinoids. Also, the accumulation of ATP generated from this enzyme may be necessary for multiple defence mechanisms as viral infection in leaf is a common phenomenon in 'Bhut Jolokia'.

A specifically expressed protein in 'Bhut Jolokia' is NACA3 (Spot no. 29). This protein is known to play important role in Na^+-dependent cellular Ca^{2+} efflux [63].

Another specifically expressed protein in 'Bhut Jolokia' is Triose Phosphate Isomerase cytosolic isoform (Spot no. 37). Triose Phosphate Isomerase plays an important role in catalyzing the inter-conversion of dihydroxyacetone-P and glyceraldehyde 3-P in the glycolytic pathway and is essential for efficient energy production [64].

Also, a Stromal 70 kDa heat shock-related protein, chloroplastic (Spot no. 107) showed 12.28 fold upregulation in 'Bhut Jolokia'. 70 kDa heat shock proteins (Hsp70s) are involved in essential cellular processes such as protein folding and protein transport across membranes and known to act as molecular chaperones; thus playing important role in the cell's response to a wide range of stress conditions [65].

One general finding from the above discussion is that major part of Bhut Jolokia proteome is involved in stress responsiveness. Stress (drought, osmotic, pathogen load etc.) is known to result in accumulation of Capsaicin. High hot and humid climatic conditions of North East Indian region is the reason of many types of stress, including viral disease, which is a common occurrence for Bhut Jolokia. These stresses might have resulted in higher capsaicin accumulation as stress is a well-known factor for higher Capsaicinoids biosyntheseis leading to a very high pungency of this chilli especially in NE region.

In addition, the proteomic analysis with 'Bhut Jolokia' fruit revealed specific expression of the gene products for ATP binding and helicase activity (spot no. 16), salt stress (spot no. 29), glycolytic enzyme (spot no. 79), protein having opposing roles during retrotranslocation (spot no. 101), multiple stress related protein (spot no. 103), and those for the citric acid cycle (spot no. 59), fatty acid synthesis (spot no. 77), transcription regulation (spot no. 94); and differential expression of proteins playing role in chloroplast development (spot no. 107) carbohydrate metabolism (spot no. 90).

Amongst the unique and differential proteins, majority had chloroplast localization (42.1%), followed by cytosol (31.5%), mitochondria (15.78%), endoplasmic reticulum (15.78%), nucleus (10.52%), and peroxisome/vacuole (5.26%). Further, the 2DE gel pattern showed that several proteins existed as multiple isoelectropherotypes for which a possibility of post translational events resulting in pI value differences cannot be ruled out. Nevertheless, it is known that 2-DE

procedure, itself, may be responsible for different isoelectropherotypes of polypeptides in 2-DE gels which may not always arise from true post translational modifications [66,67].

Also, semiquantitative analysis of the RT-PCR products of seven genes and quantitative real time PCR (qRT-PCR) analysis of two genes indicate that protein expression patterns observed in 2-DE gels are in linear correlation with significant up-regulation of the corresponding mRNAs.

The regulatory process that accounts for different accumulation levels of capsaicinoids in chili pepper fruits is not properly understood. More studies are necessary for a better understanding of the role of specific and differential proteins of 'Bhut Jolokia' towards its acute level of pungency.

Conclusions

In this study, we used proteomic techniques as a powerful tool to give some clues for elucidating the complex proteome of the hottest chilli 'Bhut Jolokia'. As a result of this investigation, out of 107 protein spots identified, twenty proteins (14 unique and 6 differential proteins) showed significant differences in expression in very pungent 'Bhut Jolokia' as compared to the less pungent *Capsicum frutescens*. This comparative analysis of proteomes from these two kinds of chilli has yielded interpretable data to look into the complex biological mechanism that might exist in the hottest chilli. It is important to note that in the absence of whole genome sequence data for these two species, the identifications rely on homologous proteins which could actually be performing discrete functions in the current model. Moreover, some of the proteins have been identified as predicted and unknown proteins, indicating novel functions operating in the fruits of this chilli plant. Out of the total twenty differentially and uniquely expressed proteins, a total of seven proteins could not be assigned any function using Pfam and COGnitor search and deserve detailed investigation to elucidate their exact role in 'Bhut Jolokia'. Detailed functional analysis of these proteins would provide further information such as that regarding direct dogmatic complex in this important source of capsaicinoids, the hottest chilli Bhut Jolokia (*C. assamicum*).

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Supplementary Files

www.omicsonline.org/0974-276X/JPB-07-s389.rar

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