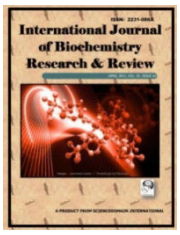

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Genome Wide Identification of Target Heat Shock Protein90 Genes and Their Differential Expression against Heat Stress in Wheat

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Research Article

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ABSTRACT

Aims: To study the genetic and transcript profiling of the genes specifying cytosolic HSP90s in *Triticum aestivum.*
Study Design: Random sampling.
Place and Duration of Study: Indian Agricultural Research Institute, New Delhi, India, between August to December, 2011.
Methodology: We include C-306 (thermotolerant) and PBW343 (thermosusceptible) cultivars of wheat for the study. Total RNA was isolated using Trizol method and gene was identified and isolated using RT-PCR. *In silico* characterization was done using different bioinformatic tools. Quantitative real time PCR was carried out using BioRad CFX96 platform and Pfaffl's method was used for the comparative change in fold expression of the gene.
Results: Here, we report cloning of an HSP90 gene from C-306 wheat cultivar having an ORF of 700 amino acids. Genome Blast identified 3 different clusters of reference sequence on chromosome no 4, 8 and 9 having LOC 100125696 and showing maximum homology with *HSP90* reported from *Triticum aestivum*. Pure amino acid composition revealed highest composition of glutamic acid followed by lysine and leucine whereas, cysteine composition was lowest. Protein characterization showed the existence of 10

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networks of coevolved amino acids. Quantitative real time PCR showed 1.5, 4.5, 5 & 7.4 fold increase in expression of HSP90 in case of C-306 compared to 2.5, 6.4, 6.9 & 5.6 fold increase in case of PBW343 at vegetative (root & shoot), pollination and milky dough stage. Multiple co-chaperones of HSP90 were observed by immunoblot assay in response to differential heat shock.

Conclusion: This investigation proves that HSP90 is one of the key components of defense mechanism in wheat against heat stress which requires the formation of co-chaperone complexes with HSP70 for its functional activity. There is a need to exploit the transcription factors associated with HSP90 and its regulation and differential expression in order to use it for developing thermotolerant wheat cultivars.

Keywords: qRT-PCR; genome profiling; wheat; fold expression; HSP90; heat shock; open reading frame; abiotic stress.

1. INTRODUCTION

Heat-shock proteins (HSPs), or stress proteins, are highly conserved and present in all organisms. Selected HSPs, also known as chaperones, play crucial roles in folding/unfolding of proteins, assembly of multi-protein complexes, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signaling, and protection of cells against stress/apoptosis. In plants, expression pattern of a number of genes modulated in response to various acute environmental changes. This altered pattern result in altered biochemical and physiological activity of the cell and developmental pursuit of the organism. When plants were exposed to high temperatures a new expression pattern was observed by the biosynthesis of heat shock proteins (HSPs) (Lindquist and Craig, 1988; Vierling, 1991). The heat-shock response in plants is similar to that of other organisms. Trimerization and activation of heat shock factors (HSFs) regulate the induction of heat shock genes. HSFs act in the promoter region of the HSPgenes through a well-defined and highly conserved heat shock element (HSE). HSP are essential components of cells and developmental processes under normal physiological conditions besides its role in heat stress. Earlier results reveal that most HSPs serve as molecular chaperones (Bukau and Horwich, 1998; Pratt et al., 2001).

HSP90 is one of the most abundant proteins in the cytoplasm, where it constitutes 1–2% of total protein levels. The essential eukaryotic chaperone HSP90facilitates the maturation of a variety of metastable protein substrates, many of which are central regulators of biological circuits. Due to HSP90's central position in numerous pathways regulating growth anddevelopment, the chaperone has emerged as a principal focus of investigation in diverse fields. The HSP90s are highly conserved and abundant cytosolic proteins in eukaryotes (Prasinos et al., 2004). Unlike other heat shock proteins, the chaperoneactivity of the HSP90 is exerted on a number of targetsubstrates or client proteins including steroid hormone receptors, cell cycle kinases, signal transduction pathway components, proteolytic machinery, and microtubule dynamics (Czar et al., 1997; Nathan et al., 1997; Garcia-Gardena et al., 1998; Holt et al., 1999; Pratt et al., 2001). However, the high abundance of HSP90 compared with thatof its client proteins, the high levels of expression in response to stress, and the existence of endoplasmic reticulum (ER), chloroplasts, and mitochondria homologues suggests that this is a very narrow view of the HSP90 cellular activity and further indicates that it may contributeto additional functions in the cytosol and organelles

under physiological or stress conditions. HSP90 acts as part of a multi-chaperone machine together with HSP70 and co-operates with a cohort of co-chaperones, including Hip (HSP70 interacting protein), Hop (HSP70/HSP90 organizing protein), p23 and HSP40 (a DnaJ homolog).

Evidence showed participation of HSP90s in different developmentally, hormonally, and morphogenetically regulated processes (Ludwig-Muller et al., 2000; Berardini et al., 2001; Muessig et al., 2002). HSP90 regulates diverse cellular functions and exerts marked effects on normal biology, disease and evolutionary processes (Taipale et al., 2010).

Plants acquire thermotolerance to lethal high temperatures if first exposed to moderately high temperature or if the temperature is increased gradually to an otherwise lethal temperature. Heat stress due to high ambient temperatures is a serious threat to crop production worldwide (Hall, 2001). Immediately after exposure to high temperatures and perception of signals, changes occur at the molecular level altering the expression of genes and the accumulation of transcripts, thereby leading to the synthesis of stress-related proteins as a stress tolerance strategy (Iba, 2002). Expression of heat shock proteins (HSPs) is known to be an important adaptive strategy in this regard (Feder and Hoffman, 1999). HSC70/HSP90 machinery is important for stomatal closure and serves essential functions in plants to integrate signals from their biotic and abiotic environments (Clement et al., 2011). Heat shock protein 90 (HSP90) molecular chaperones play important roles in plant growth and responses to environmental stimuli. However, little is known about the genes encoding HSP90s in common wheat. Here, we report genetic and transcript analysis of the genes specifying cytosolic HSP90s in this species.

2. MATERIALS AND METHODS

2.1 Plant Materials

HSP90 gene expression was studied in two different genotypes of *Triticum aestivum,* i.e., thermotolerant (C-306) and susceptible (PBW343) cultivars. Seeds were collected from Division of Genetics, Indian Agricultural Research Institute (IARI) and sown in Phytotron under regulated conditions.

2.1.1 Differential heat shock treatment

Wheat seedlings were grown in a 3:1 soil to per liter mixture in a growth chamber inside phytotron under controlled conditions (22°C, 16h light/8h dark cycle). Fourteen days-old (14 d old) wheat plants were subjected to differential heat shock treatment of 30°, 35° and 40°C for 2 h. Plants of the same age that were not exposed to thermal stress were included as control. Samples consisted of leaf tissues collected from 3-5 plants for each of the three categories of treated and control plants. Samples were immediately frozen in liquid nitrogen and maintained at -80°C.

2.2 Isolation and Cloning of HSP90 from Wheat

After an *in silico* identification of conserved sequence regions of HSP90 genes in cereals, a pair of degenerated primers was designed for PCR amplification of the corresponding gene sequences in wheat. Total RNA was isolated from 14 days old germinating wheat seedlings by Trizol method (Invitrogen). cDNA was synthesized using oligod T primer (RevertAid^{TM} H minus First Strand cDNA Synthesis Kit, Fermentas) and were used for PCR amplification of

HSP90 gene by using the degenerate primers (Table 1). The amplified products were cloned in pGEMT easy vector (Promega) and sequenced using M13 forward and reverse primers.

Table 1. List of primers used for quantitative real time (qRT-PCR) amplification of Heat Shock Protein 90 (HSP90) gene in wheat.

Primers	Sequence (5'-3')	Tm (°C)
Act- F_q	5'-GTTGCTCCAGAAGAGCATCC-3'	60.5°C
Act-R_q	5'-ACATACATGGCGGGAACATT-3'	56.4°C
HSP-90F_q	5'-TGATGATGGGTGGACTGCCAACAT-3'	62.7°C
HSP-90R_q	5'-TCTCGAAGAGCAGCATCACAAGGT-3'	62.7°C
HSP-90F	5'-TCCCGCACGCTTCTCCT-3'	57.2°C
HSP-90R	5'-AACTGTTCCACGAGTACCACA-3'	59.4°C

† Act-Actin; HSP-Heat Shock Protein; F_q & R_q-Forward and Reverse primers for qRT-PCR

2.3 Genome Blast and *In Silico* Analysis of HSP90 Genes

The isolated HSP90 gene was submitted in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) using Bankit (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). The translational characterization of isolated genes was carried out using Expasy tool (http://expasy.org/tools/). Open Reading Frame of the isolated gene was characterized using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Different HSP90 gene reported from plant and non-plant sources were retrieved from GenBank and were aligned along with HSP90 isolated in the present investigation using the Clustal W alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). NCBI genome blast was used to characterize the location and existence of isolated HSP90 gene on the chromosomes of related species.

2.4 RNA Extraction and Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted by Trizol method (Invitrogen) and quantified by Qubit™ 2.0 fluorometer (Invitrogen, UK). RNA integrity was verified in1.2% agarose gels. First strand cDNA synthesis was performed using oligod T (RevertAid™ H minus First Strand cDNA Synthesis Kit, Fermentas) according to the manufacturer's instructions. First-strand cDNA was diluted to a final concentration of 1.0 µg/µl. Primers for RT-qPCR reactions were designed from the deduced sequence corresponding to the wheat HSP90 gene using Prime 3 primer designing software (Premier Biosoft, USA) (Table 1). For each stress condition as well as for controls, expression measurements were performed using duplicate biological replications and three technical replications. Quantitative PCR was performed in 25µl reactions using genes specific primers, 1µl of cDNAas template and the SYBR GreenER qPCR SuperMix Universal (Invitrogen, UK). Reactions were performed on the CFX96 Real-Time PCR system (BioRad, UK). The thermal profile for qPCR was: 3 min at 95°C, followed by 35 cycles each consisting of 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 3 % agarose gel. The expression levels of wheat actin gene were used as internal standards for normalization of cDNA template quantity using actin-specific primers (accession No. AF282624) (Table 1). For each stress condition as well as for controls, expression measurements were performed using duplicate biological replications and three technical replications. Controls with no cDNA template were also included in qPCR analysis. Data analysis was performed using the software provided

by BioRad, UK. The Comparative Ct (2-ΔΔCt) method was used to calculate the changes in gene expression as a relative fold difference between an experiment and calibrator sample.

2.5 Immunoblot Analysis of Heat Shock Protein

Samples collected at different developmental stages were ground to fine powder in liquid nitrogen. The powder was transferred immediately to a 10-ml tube containing extraction buffer (50 mM phosphate, pH 7.0, 0.2% (v/v) Triton-X-100, 7 mMβ- mercaptoethanol and 5 mM ascorbic acid). The homogenate was centrifuged at 12,000 rpm for 15 min at 4℃. The supernatant was transferred to a fresh tube and incubated at 100℃ for 10 min, then the tube was transferred to ice for 5 min. The heat-treated supernatant was centrifuged at 12,000 rpm for 15 min, and 50–100 µl of supernatant was transferred to new tubes for direct use or storage at –80℃. Concentrations of heat-stable proteins were determined according to the Bradford protocol. SDS–PAGE was performed with a discontinuous buffer system, as described by Laemmli (1970). Protein samples in 1X SDS gel-loading buffer were denatured by heating at 95℃ for 5 min before loaded into the gel. 15µg of protein samples were separated electrophoretically on low range SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) microporous membranes using the semi dry blotter (Invitrogen). The western blotting procedures were carried out according to Mazhar and Basha (2002) using anti-HSP90 antibody and mice anti-rabbit IgG antibody conjugated with alkaline phosphatase. Fresh-developing buffer (100 µl of NBT solution and 100 µl of BCIP solution in 10 ml of alkaline phosphatase buffer) was used for membrane staining, and the reaction was stopped by washing the membrane in double distill water.

3. RESULTS AND DISCUSSION

3.1 Sequence Identification of HSP90Gene

An amplicon of 2323bp was amplified from C-306 cultivar of wheat by RT-PCR and cloned in maintenance vector. BLAST nhomology analysis showed that the amplified sequence has 96% resemblance with HSP90 gene reported from *Triticum aestivum* L. The gene was submitted to NCBI GenBank with accession number JN052206.

3.2 *In Silico* Characterization

3.2.1 Clustal W alignment and phylogeny analysis

In order to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences, amino acid sequences of HSP90 reported from plant sources (*Triticum aestivum*, accession no. JN052206; *Hordeum vulgare,* accession no. AY325266; *Oryza sativa,* accession no. AB111818; *Zea mays,* accession no. NM_001177; *Arabidopsis thaliana,* accession no. AY081382, AY062832 and AY070031) were retrieved from GenBank and were analyze for mismatch pairing since they diverge from one another (Fig. 1). Clustal W alignment showed that the sequences were highly conserved with few point mutations. Such conserved sequence motifs can be used in conjunction with structural and mechanistic information to locate the catalytic active sites of enzymes. Alignments obtained in the present investigation were used to aid in establishing evolutionary relationships by constructing phylogenetic trees (Fig. 1). The sequence alignments were used for the construction and interpretation of phylogenetic trees, which was further used to classify the evolutionary relationships between homologous HSP90 genes represented in the genomes of divergent *species.*HSP90 reported from *Triticum*

aestivum and *Hordeum vulgare* has high sequence identity which suggests that the sequences in question have a comparatively young most recent common ancestor, while sequences reported from *Arabidopsis thaliana* has a very low identity with HSP90 from *Triticum aestivum* which suggests that the divergence is more ancient (Fig. 2).

Phylogenetic relationships of HSP90, a less conserved and less widely distributed general chaperone, is strikingly incongruent with canonical patterns of endosymbiotic ancestry. It appears that HSP90 of chloroplasts derives from the endoplasmic reticulum specific isoform while mitochondrial HSP90 homologs affiliate with a bacterial lineage other than α subdivision of proteobacteria (Emelyanov, 2002).

```
JN052206          QPELFIHIIPDKATNTLTIVDSGIGMTKSDLVNNLGTIARSGTKEFMEALAAGADVSMIG 120
AY325266          QPELFIHIIPDKATSTLTIVDSGIGMTKSDLVNNLGTIARSGTKEFMEALAAGADVSMIG 120
AB111810          QPELFIHIVPDKASNTLSIIDSGVGMTKSDLVNNLGTIARSGTKEFMEALAAGADVSMIG 120
NM_001177009      QPELFIHIVPDKANNTLTIIDSGIGMTKSDLVNNLGTIARSGTKEFMEALAAGADVSMIG 120
AY081302          QPELFIHIIPDKTNNTLTIIDSGIGMTKADLVNNLGTIARSGTKEFMEALAAGADVSMIG 119
AY070031          QPELFIHIIPDKTNNTLTIIDSGIGMTKADLVNNLGTIARSGTKEFMEALAAGADVSMIG 119
AY062832          QPELFIHIIPDKTNNTLTIIDSGIGMTKADLVNNLGTIARSGTKEFMEALAAGADVSMIG 119
                  ********:***:..**:*:***:****:******************************

JN052206          EEKEEKEKKKKKIKEVSHEWNLVNKQKPIWMRKPEEINKEEYAAFYKSLTNDWEEHLAVK 300
AY325266          EEKEEKEKKKKKIKEVSHEWNLVNKQKPIWMRKPEEINKEEYAAFYKSLTNDWEEHLAVK 300
AB111810          EEKEEKEKKKKKIKEVSHEWNVMNKQKPIWLRKPEEITKEEYAAFYKSLTNDWEEHLAVK 300
NM_001177009      DEKEEKEKKKKKIKEVSHEWQLVNKQKPIWMRKPEEITKEEYAAFYKSLTNDWEEHLAVK 299
AY081302          EEKEKEEKKKKKIKEVSHEWDLVNKQKPIWMRKPEEINKEEYAAFYKSLSNDWEEHLAVK 298
AY070031          EEKEKEEKKKKKIKEVSHEWDLVNKQKPIWMRKPEEINKEEYAAFYKSLSNDWEEHLAVK 298
AY062832          EEKEKEEKKKKKIKEVSHEWDLVNKQKPIWMRKPEEINKEEYAAFYKSLSNDWEEHLAVK 298
                  :***::**************:::*******:******.***********:**********

JN052206          HFSVEGQLEFKAVLFVPKRAPFDLFDNKKKANNIKLYVRRVFIMDNCEELIPEYLSFVKG 360
AY325266          HFSVEGQLEFKAVLFVPKRAPFDLFDNKKKANNIKLYVRRVFIMDNCDELIPEYLSFVKG 360
AB111810          HFSVEGQLEFKAILFVPKRAPFDLFDTRKKQNNIKLYVRRVFIMDNCEELIPEWLSFVKG 360
NM_001177009      HFSVEGQLEFKAVLFVPKRAPFDLFDTRKKQNNIKLYVRRVFIMDNCEELIPEWLSFVKG 359
AY081302          HFSVEGQLEFKAILFVPKRAPFDLFDTKKKPNNIKLYVRRVFIMDNCEDIIPEYLGFVKG 358
AY070031          HFSVEGQLEFKAILFVPKRAPFDLFDTKKKPNNIKLYVRRVFIMDNCEDIIPEYLGFVKG 358
AY062832          HFSVEGQLEFKAILFVPKRAPFDLFDTKKKPNNIKLYVRRVFIMDNCEDIIPEYLGFVKG 358
                  ************:************.:** ****************:::***:*.****

JN052206          LEKLKKKGYEVIYMVDAIDEYAIGQLKEFEGKKLVSATKEGLKLDESEDEKKKQEELKEK 540
AY325266          LEKLKKKGYEVIYMVDAIDEYAIGQLKEFEGKKLVSATKEGLKLDESEDEKKKQEELKEK 540
AB111810          LEKLKKKGYEVLYMVDAIDEYAVGQLKEFEGKKLVSATKEGLKLDESEDEKKRQEELKEK 540
NM_001177009      LEKLKKRGYEVLFMVDAIDEYSIGQLKEFEGKKLVSATKEGLKLDESEDEKKRKEELKEK 539
AY081302          LEKLKKKGIEVLYMVDAIDEYAIGQLKEFEGKKLVSATKEGLKLDETEDEKKKKEELKEK 538
AY070031          LEKFKKKGIEVLYMVDAIDEYAIGQLKEFEGKKLVSATKEGLKLDETEDEKKKKEELKEK 538
AY062832          LEKLKKKGIEVLYMVDAIDEYAIGQLKEFEGKKLVSATKEGLKLDETEDEKKKKEELKEK 538
                  ***:**:* **::********::************************:*****::******

JN052206          MSSKKTMEINPENAIMDELRKRADADKNDKSVKDLVMLLFETSLLTSGFSLEDPNTFGTR 660
AY325266          MSSKKTMEINPENAIMDELRKRADADKNDKSVKDLVMLLFETSLLTSGFSLEDPNTFGTR 660
AB111810          MSSKKTMEINPENSIMDELRKRADADKNDKSVKDLVMLLFETALLTSGFSLEDPNTFGTR 660
NM_001177009      MSSKKTMEINPENAIMEELRKRAEADKNDKSVKDLVMLLFETALLTSGFSLDDPNTFGSR 659
AY081302          MSSKKTMEINPENSIMDELRKRADADKNDKSVKDLVLLLFETALLTSGFSLDEPNTFGSR 658
AY070031          MSSKKTMEINPENSIMDELRKRADADKNDKSVKDLVLLLFETALLTSGFSLDEPNTFGSR 658
AY062832          MSSKKTMEINPENSIMDELRKRADADKNDKSVKDLVLLLFETALLTSGFSLDEPNTFGSR 658
                  *************:**:******:************:*****:********::******:*
```

Fig. 1. Clustal W alignment of different HSP90 reported from *Triticum aestivum*, accession no. JN052206; *Hordeum vulgare,* accession no. AY325266; *Oryza sativa,* accession no. AB111818; *Zea mays,* accession no. NM_001177; *Arabidopsis thaliana,* accession no. AY081382, AY062832 and AY070031

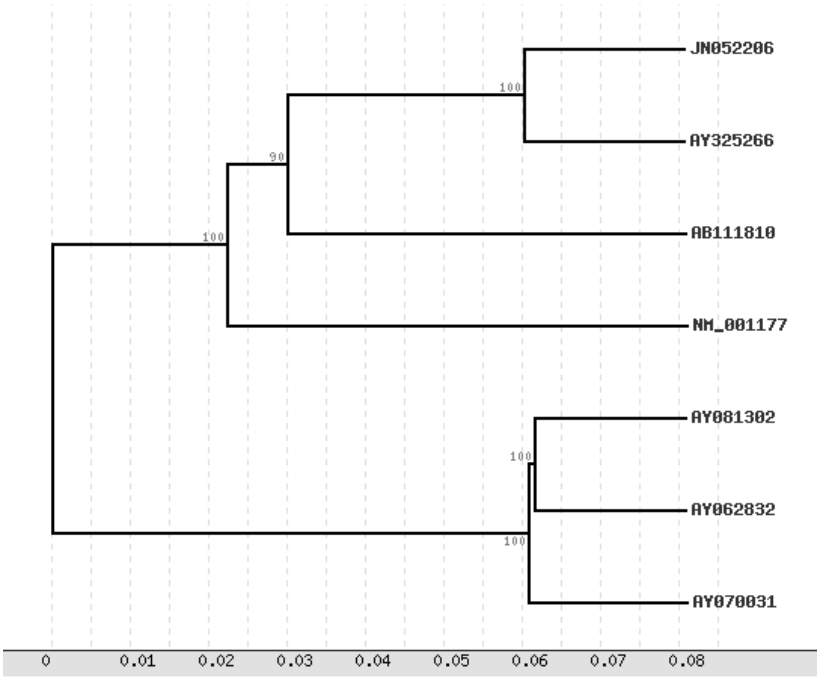

Fig. 2. Phylogeny tree analysis of different HSP90 sequences isolated from *Triticum aestivum*, accession no. JN052206; *Hordeum vulgare,* accession no. AY325266; *Oryza sativa,* accession no. AB111818; *Zea mays,* accession no. NM_001177; *Arabidopsis thaliana,* accession no. AY081382, AY062832 and AY070031

3.2.2 Genome blast and chromosomal mapping

The genome blast was done to locate the location of reference sequence of HSP90 in *H. vulgare* since the wheat genome is partially sequenced. Genome Blast identified 3 different clusters of reference sequence on chromosome no 4, chromosome no 8 and chromosome no 9 having LOC 100125696 and showing maximum homology with HSP90 reported from *Triticum aestivum* (Fig. 3). The clusters transcript were compare with that of reference HSP90 proteins and the best hits with reference to each cluster are shown in table 2. The approximate expression pattern inferred from EST sources was observed in inflorescence, root, leaves and whole plant respectively. The EST sequences observed showed similarity to HSP90 protein reported from *Triticum aestivum* (even after translation).

In the plant *Arabidopsis thaliana*, HSP90 homologs are encoded by seven different genetic loci. Of these, one is expressed in the endoplasmic reticulum (HSP90.7), one in the mitochondrion (HSP90.6), one in the chloroplast (HSP90.5), and four in the cytosol. The gene encoding one cytosolic protein (HSP90.1/At5g52640) is highly stress-inducible, whereas the other three (HSP90.2/At5g56030, HSP90.3/At5g56010, and HSP90.4/At5g56000) are constitutively expressed and are the products of very recent duplication events. Two of the organelle specific HSP90s, have been shown to affect plant development. Three groups of homoeologous genes (TaHsp90.1, TaHsp90.2 and TaHsp90.3), encoding three types of cytosolic HSP90, were isolated. The loci containing TaHsp90.1, TaHsp90.2 and TaHsp90.3 genes were assigned to groups 2, 7 and 5 chromosomes, respectively (Xuejun et al., 2011).

Table 2. List of transcripts showing maximum homology with reference sequence (TaHSP90) observed in different clusters on chromosome no 4, 7 and 8 of *Hordeum vulgare*

Chromosome no 4				
Best Hits		**Species**	**Id(%)**	**Len(aa)**
XP_002460433.1	Hypothetical protein SORBIDRAFT_02g028050	*S. bicolor*	89.5	257
NP_001062159.1	Os08g0500700	*O. sativa*	89.1	257
NP_200076.1	ATHSP90.1 (HEAT SHOCK PROTEIN 90.1); ATP binding / unfolded protein binding	*A. thaliana*	85.6	257
XP_001695264.1	Heat shock protein 90A	*C. reinhardtii*	76.5	255
XP_323482.1	Hypothetical protein ((AL513463) heat shock protein 80	*N. crassa*	67.3	263
NP_001086624.1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	*X. laevis*	67.3	269
NP_005339.3	Heat shock protein HSP 90-alpha isoform 2	*H. sapiens*	65	274
NP_034610.1	Heat shock protein HSP 90-alpha	*M. musculus*	64.7	275
NP_013911.1	Cytoplasmic chaperone of the Hsp90 family, redundant in function and nearly identical with Hsp82p	*S. cerevisiae*	64.5	211
NP_523899.1	Heat shock protein 83	*D. melanogaster*	63.3	270
NP_001038538.1	Heat shock protein 90-alpha 2	*D. rerio*	60.5	268
NP_506626.1	Abnormal DAuer Formation family member (daf-21)	*C. elegans*	59.3	204

Table 2 continues……

Chromosome no 8 **Best Hits and Hits from model organisms**		**Species**	**Id(%)**	**Len(aa)**
XP_002460433.1	Hypothetical protein SORBIDRAFT_02g028050	*S. bicolor*	89.5	257
NP_001062159.1	Os08g0500700	*O. sativa*	89.1	257
NP_200076.1	ATHSP90.1 (HEAT SHOCK PROTEIN 90.1); ATP binding / unfolded protein binding	*A. thaliana*	85.6	257
XP_001695264.1	Heat shock protein 90A	*C. reinhardtii*	76.5	255
XP_323482.1	Hypothetical protein ((AL513463) heat shock protein 80	*N. crassa*	67.3	263
NP_001086624.1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	*X. laevis*	67.3	269
NP_005339.3	Heat shock protein HSP 90-alpha isoform 2	*H. sapiens*	65	274
NP_034610.1	Heat shock protein HSP 90-alpha	*M. musculus*	64.7	275
NP_013911.1	Cytoplasmic chaperone of the Hsp90 family, redundant in function and nearly identical with Hsp82p,and together they are essential; expressed constitutively at 10-fold higher basal levels than HSP82 and induced 2-3 fold by heat shock	*S. cerevisiae*	64.5	211
NP_523899.1	Heat shock protein 83	*D. melanogaster*	63.3	270
NP_001038538.1	Heat shock protein 90-alpha 2	*D. rerio*	60.5	268
NP_506626.1	Abnormal DAuer Formation family member (daf-21)	*C. elegans*	59.3	204

Table 2 continues……

Chromosome no 9 **Best Hits and Hits from model organisms**		**Species**	**Id(%)**	**Len(aa)**
XP_002460433.1	Hypothetical protein SORBIDRAFT_02g028050	*S. bicolor*	89.5	257
NP_001062159.1	Os08g0500700	*O. sativa*	89.1	257
NP_200076.1	ATHSP90.1 (HEAT SHOCK PROTEIN 90.1); ATP binding / unfolded protein binding	*A. thaliana*	85.6	257
XP_001695264.1	Heat shock protein 90A	*C. reinhardtii*	76.5	255
XP_323482.1	Hypothetical protein ((AL513463) heat shock protein 80	*N. crassa*	67.3	263
NP_001086624.1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	*X. laevis*	67.3	269
NP_005339.3	Heat shock protein HSP 90-alpha isoform 2	*H. sapiens*	65	274
NP_034610.1	Heat shock protein HSP 90-alpha	*M. musculus*	64.7	275
NP_013911.1	Cytoplasmic chaperone of the Hsp90 family, redundant in function and nearly identical with Hsp82p,and together they are essential; expressed constitutively at 10-fold higher basal levels than HSP82 and induced 2-3 fold by heat shock	*S. cerevisiae*	64.5	211
NP_523899.1	Heat shock protein 83	*D. melanogaster*	63.3	270
NP_001038538.1	Heat shock protein 90-alpha 2	*D. rerio*	60.5	268
NP_506626.1	Abnormal DAuer Formation family member (daf-21)	*C. elegans*	59.3	204

3.2.3 Protein characterization of HSP90

HSP90 gene isolated in present investigation has an ORF of 700 aa with start codon at 62bp and stop codon at 2164bp. Clustal W showed variability in HSP90 sequence and phylogeny of HSP90 gene of wheat sequences collected from GenBank classified it into four different subgroups. HSP90 belongs to subgroup having the same origin as that of *Triticum aestivum* (accession no. GQ240780.1 and GQ40779.1).

Pure amino acid compositions (AAC) of different HSP90 proteins reported from wheat, maize and rice were carried out in order to understand the co-evolution of amino acid and protein interaction. In all most all the proteins, glutamic acid percentage was very high followed by lysine and leucine whereas,cysteine composition was lowest (Fig. 4a). The protein of isolated HSP90 were characterize for phosphorylating sites using NetPhos 2.0 server (Fig. 4b). Numerous sites with serine (24 sites), threonine (7 sites) and tyrosine (9 sites) were predicted as potential phosphorylating sites all over the amino acid sequence of HSP90. The site at position 169 with sequence -EKEISDDED- has highest activity for serine phosphorylation, position 113 with sequence -VTRDTSGEQ- for threonine and position 231 with sequence –NKEEYAAFY- for tyrosine. PgHSP90 isolated from *Pennesiteum typhoides* possesses the five-conserved amino acid signature sequence motifs characteristic of the HSP90 family and a C-terminus MEEVD penta-peptide characteristic of the cytosolic HSP90 isoform (Reddy et al., 2010). The requirement of many principal regulatory proteins for HSP90 renders entire pathways sensitive to decreases in its function (Salathia et al., 2007).

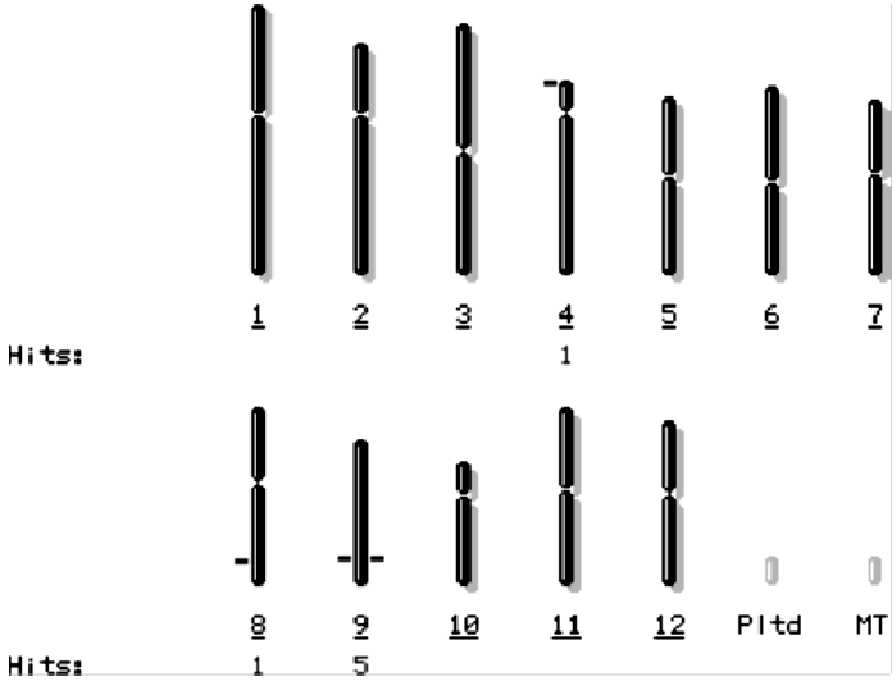

Fig. 3. Chromosomal mapping of *Hordeum vulgare* with location of HSP90 mRNA align to genomic sequence using Genomic Blast (NCBI). HSP90 reference sequence was observed on chromosome no 4, 8 (one HSP90) and chromosome no 9 (five HSP90)

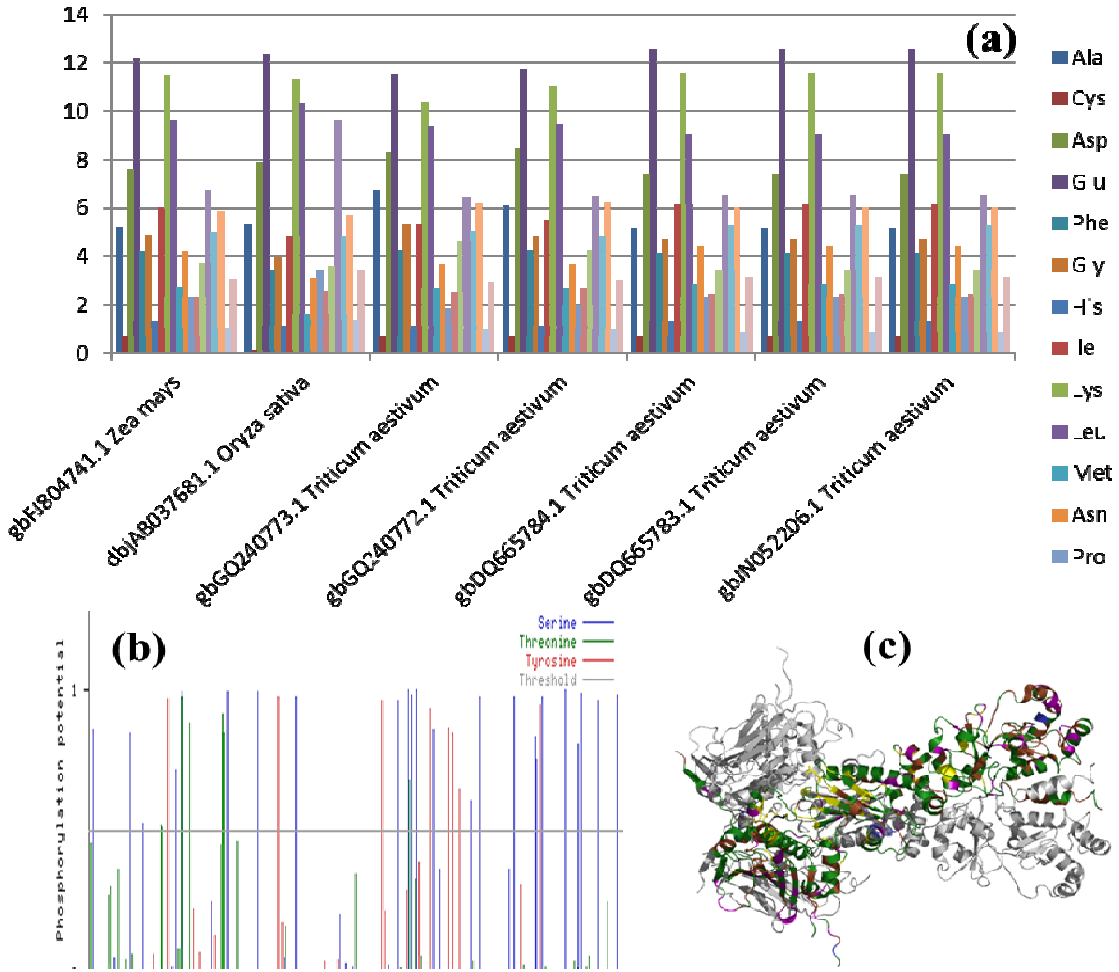

Fig. 4. *In silico* characterization of Heat Shock Protein 90 (Accession no JN052206) (a) Pure amino acid compositions, (b) Predicted potential phosphorylating sites using NetPhos 2.0 server and, (c) Co-evolving pair of amino acids as depicted on the 3D structure of the HS protein 90 isolated in present investigation

3.2.4 Co-evolving amino acid in HSP90 protein

Communication between distant sites often defines the biological role of a protein. Amino acid long-range interactions are as important in binding specificity, allosteric regulation and conformational change as residues directly contacting the substrate (Fares et al., 2006). The maintaining of functional and structural coupling of long-range interacting residues requires coevolution of these residues. InterMap3D 1.3 server analysis revealed 10 different coevolving amino acid pairs (Table 3). Coevolved residues often play a major biological role in the protein, and the nature of their interactions might be multiple, spanning among binding specificity, allosteric regulation and conformational change of the protein. Fares et al. (2006) research on coevolution analysis and functional data for heat-shock proteins, HSP90 and GroEL, highlight that almost all detected coevolving sites are functionally or structurally important and he proposed that new amino acid sites are important for inter-domain functional communication.

Table 3. List of co-evolving amino acid pairs based on MI/Entropy in HSP90 isolated from *Triticum aestivum*

Position 1	Position 2	Prediction	Score	Distance
96	99	co-evolution	12.69	6.949 °A
96	282	co-evolution	12.69	43.867 °A
96	286	co-evolution	12.69	42.188 °A
99	282	co-evolution	12.69	41.309 °A
99	286	co-evolution	12.69	40.069 °A
282	286	co-evolution	12.69	6.438 °A
301	378	co-evolution	7.13	24.913 °A
171	172	co-evolution	6.63	3.800 °A
120	490	co-evolution	6.17	62.853 °A
482	564	co-evolution	6.02	8.123 °A

3.3 Analysis of Fold Change in Expression of HSP90 Gene

The real time expression profiling of HSP90 gene in root under heat shock of 42℃ for 2h at seedling stage showed 1.5 fold increase of expression in C-306 compared to 2.6 fold increases in PBW343 (Fig. 5a). The transcript level in roots was approximately three fold less than in leaves but light–dark treatment did not charge TaHSC70 expression in wheat (Kang et al., 2011). Relative expression profiling of leaf samples collected at seedling stage showed 4.5 fold increase in the expression of HSP90 in C-306 compared to 6.4 fold increase in case of PBW343 (Fig. 5b). Following heat shock of wheat seedlings at 40℃, TaHSC70 expression increased in leaves of etiolated seedlings but remained stable at the same level in green seedlings wheat (Kang et al., 2011). The result showed 5 fold increases in the expression of HSP90 gene in C-306 compare to 6.9 fold increases in case of PBW343 in response to heat shock of 42℃ for 2 h during the pollination stage (Fig. 5c). The qRT-PCR showed 7.4 and 5.6 fold increase in the expression of HSP90gene in C-306 and PBW343 at milky dough compared to control (Fig. 5d).

TaHsp90.1 genes exhibited higher transcript levels in the stamen than in the leaf, root and culm. TaHsp90.2 and TaHsp90.3 genes were more ubiquitously transcribed in the vegetative and reproductive organs examined (Zhang et al., 2011). The quantitative up-regulation of *PgHsp90* gene expression positively correlates in response to different stresses to meet the additional demand for protein folding support (Reddy et al., 2010). Semi-quantitative Real

Time expression profiling of HSP90 in C-306 (tolerant) and PBW343 (susceptible) wheat cultivars at different stages of growth against HS (42℃ for 2h) showed marked change in the expression of HSP90 gene on gel (Fig. 6).

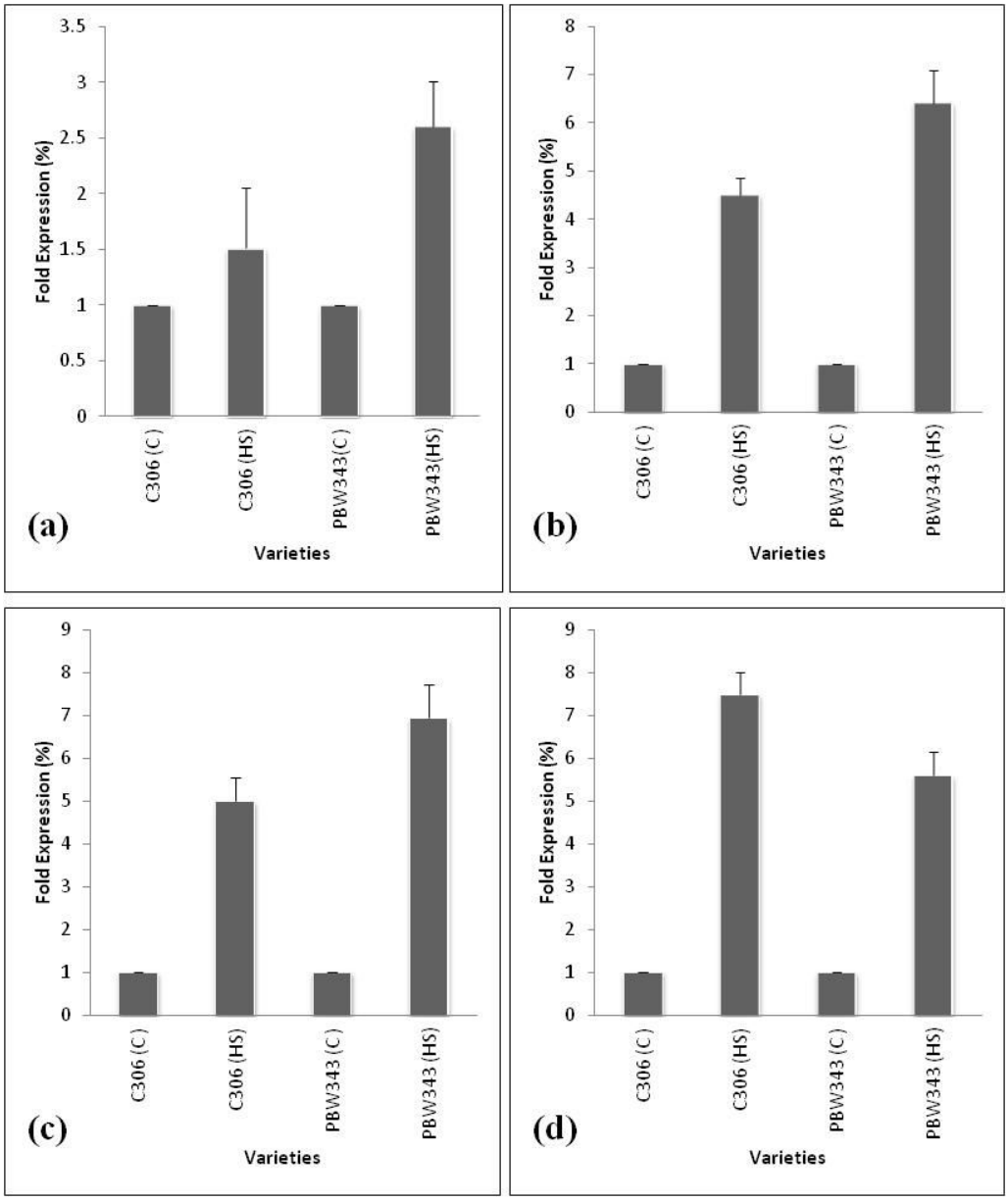

Fig. 5. Comparative change in fold expression of HSP90 gene in thermotolerant (C-306) and susceptible (PBW343) cultivars of wheat under different stages of growth (a) vegetative root (b) vegetative leaves (c) pollination stage and, (d) milky dough stage, against heat shock of 42℃ for 2h (HS)

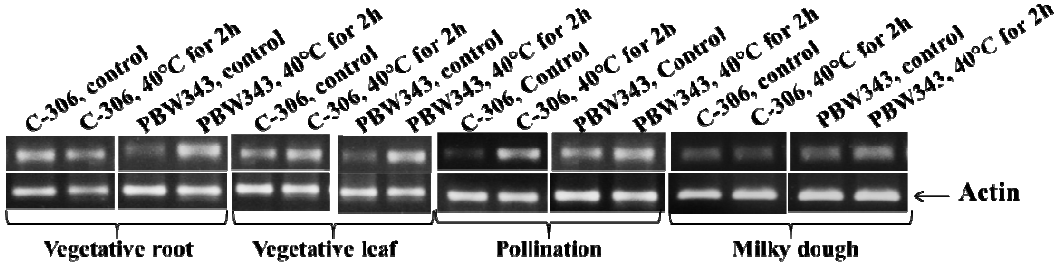

Fig. 6. Semi-quantitative Real Time expression profiling of HSP90 in C-306 (tolerant) and PBW343 (susceptible) wheat cultivars at different stages of growth against heat shock of 42℃ for 2h (HS), the expression of actin gene was used as endogenous control

Differential heat shock treatment (30°, 35° and 40℃ for 2h) showed 1.8, 2.1 and 2.7 fold increase in expression of HSP90 in C-306 cultivar and 1.9, 2.6 and 4.1 fold increases in case of PBW343 against HS (Fig. 7). Deficiency in the expression of chaperones often results in increased thermo sensitivity or death of the organism even under normal growth conditions. HSP90 is having an ATP domain having the main function of phosphorylation of protein and is involved in signaling pathways. An increase in the expression of HSP90 mRNA level was also observed against 5℃ cold stress treatment (Krishna et al., 2001). Transient expression of HSP90 in TU8 mutant protoplasts of *Arabidopsis* increased their survival rate at higher temperatures to near equivalent that of wild-type protoplasts (Ludwig Muller et al., 2000). Eukaryotic genomes contain genes encoding constitutively expressed and inducible cytosolic HSP90 (Taipale et al., 2010). Semi-quantitative Real Time expression profiling of HSP90 in seedlings of C-306 (tolerant) and PBW343 (susceptible) wheat cultivars under differential heat shock treatment of 30°, 35° and 40℃ for 2 h showed a continuous increase in the fold expression of HSP90 (Fig. 8). In most eukaryotes, HSP90 expression approximately doubles in response to environmental stress.

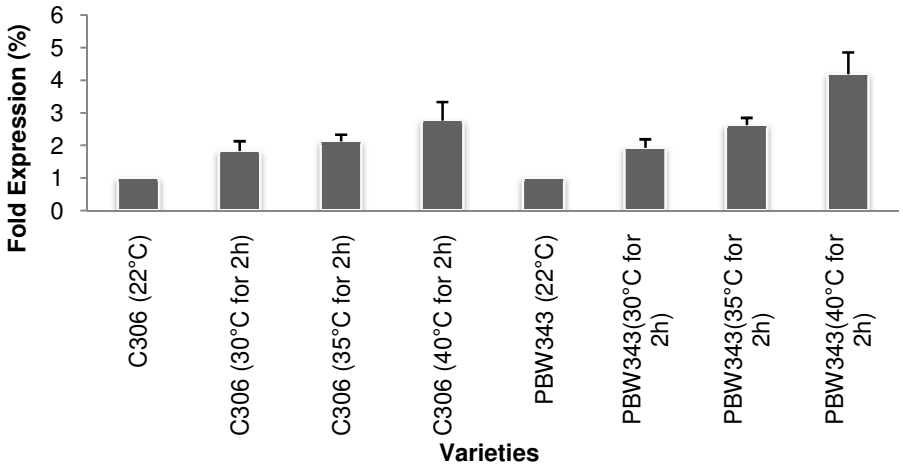

Fig. 7. Change in the expression profile of HSP90 genes in C-306 (thermotolerant) and PBW343 (susceptible) cultivars of wheat under differential heat shock treatment of 30°, 35° and 40℃ for 2 h, Actin gene was used as endogenous control

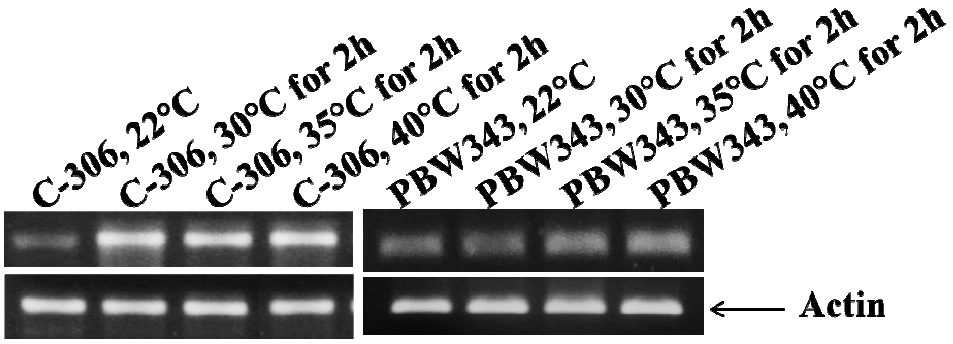

Fig. 8. Semi-quantitative Real Time expression profiling of HSP90 in seedlings of C-306 (tolerant) and PBW343 (susceptible) wheat cultivars under differential heat shock treatment of 30°, 35° and 40°C for 2 h, The expression of actin gene was used as endogenous control

3.4 Immunoblot Analysis Using HSP90 Specific Antibodies

Samples collected from C-306 and PBW343 at different stages of growth (Vegetative, pollination and milky dough) were used for immune blot analysis using anti-HSP90 antibodies synthesized in rabbit (Fig. 9). The presence of HSP90 was not observed during vegetative stage in both the cultivars. During pollination and milky dough stage an increase in the expression of HSP90 was observed with prominent blots in case of tolerant compared to faint blots in susceptible cultivar which contradict our earlier result where the expression was observed more in case of susceptible cultivar.But then, the change in expression (qRT-PCR) and the difference in blot (western blotting) are on par. The presence of HSP90 at pollination and milky dough stage confirms our earlier finding that HSP90 plays a very prominent role in thermotolerance other than the signaling role.

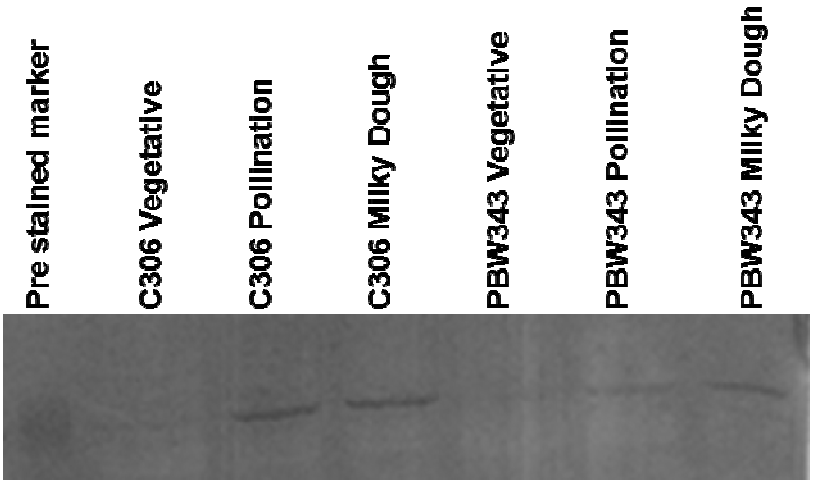

Fig. 9. Western blot analysis of C-306 (tolerant) and PBW343 (susceptible) cultivars of wheat at different stages of growth (vegetative, pollination and milky dough) using anti-HSP90 specific antibody

In case of C-306 against differential HS showed numerous complexes or co-chaperones of HSP90 within the range of 87 to 98 KDa (Fig. 10). The maximum numbers of complexes (seven) were observed against heat shock of 35°C for 2 h. Similarly, four different complexes were observed in case of PBW343 against heat shock of 40°C for 2 h. In present investigation, even though the primary antibody used was Mab-HSP90, but then we got the band in the range of ~87 KDa to ~98 KDa which are predicted to be a dissociated multi protein complexes of HSP90 and HSP70 bounded to Hop.

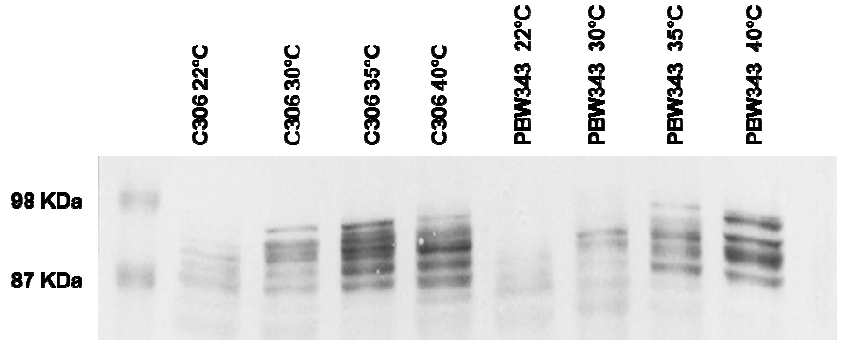

Fig. 10. Immunoblot analysis of wheat cultivars using anti-HSP90 specific antibodies in response to differential heat shock treatment of 30°, 35° and 40°C for 2 h

4. CONCLUSION

In conclusion, HSP90 plays very important role in signaling as well as thermo tolerance in different crops. An HSP90 gene isolated in present investigation showed same phylogenetic origin as that of HSP90 reported from *Hordeum vulgare.* Genome Blast identified 3 different clusters of reference sequence on chromosome no 4, chromosome no 8 and chromosome no 9. Fine analyses of families of protein sequences reveal the existence of 10 networks of coevolved amino acids. Abundance of HSP90 transcript was observed during pollination and milky dough stage in both thermotolerant and susceptible cultivars. The extent of diversity observed in case of HSP90 can be used by breeders to characterize different germplasm of crops for their abiotic stress tolerance capacity. Further analysis of the HSP90 promoter elements may lead to better understanding of the regulatory mechanisms controlling the tissue specific expression of HSP90 gene in order to use it for enhancing the thermotolerance capacity of plant.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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