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Heat shock protein based SNP marker for terminal heat stress in wheat (*Triticum aestivum* L.)

Deepa Garg[1], Sindhu Sareen[1], Sunita Dalal[2], Ratan Tiwari[1], Rajender Singh[1]*

[1]Directorate of Wheat Research, Karnal-132001, INDIA
[2]Kurukshetra University, Kurukshetra, INDIA

***Corresponding author: rajenderkhokhar@yahoo.com**

Abstract

High temperatures during grain filling period of wheat adversely affect the plant growth, yield and grain quality in many regions of world. Tolerance to heat stress is complex phenomenon and controlled by multiple genes imparting a number of physiological and biochemical changes. Attempts were made to identify single nucleotide polymorphism (SNP) and to differentiate the heat tolerant and heat susceptible genotypes of wheat using heat shock protein (*HSP16.9*) as the target gene. DNA fragment covering a partial sequence of *Triticum aestivum* L. *HSP16.9*, were amplified from heat tolerant genotype (K7903) and heat susceptible genotype (RAJ4014), and subsequently analyzed for the presence of SNP. One SNP was found between these genotypes and the analysis of amino acid sequence showed that the base transition (A/G) positioned at 31 amino acid resulted in missense mutation from aspartic acid to aspargine residue. Allele specific primers based on SNP were designed to screen the other heat tolerant and susceptible genotypes. Single-marker analysis explained 29.89% and 24.14% phenotypic variation for grain weight per spike and thousand grain weight respectively. This is the first report of HSP derived SNP marker associated with terminal heat stress in wheat which can be used by the breeders for improving tolerance to high temperatures in wheat breeding programmes.

Keywords: Single nucleotide polymorphism, heat stress, heat shock protein, wheat.
Abbreviations: HSP- Heat shock protein; SNP- single nucleotide polymorphism; DH- days to heading; DA- days to anthesis; DM- days to physiological maturity; GFD- grain filling duration; GN- grain number per spike; GW- grain weight per spike; TGW- thousand grain weight; YLD- yield; GGR- grain growth rate; HSI- heat susceptibility index.

Introduction

Climate change is set to increase the frequency and severity of environmentally limited production as global warming will cause more frequent extreme temperature events. The global temperature increased by 0.74^0C between 1906 and 2005 (IPCC 2007a). Intergovernmental Panel on Climate Change projected that temperature increase by the end of this century is expected to be in the range 1.8 to 4.0^0C (IPCC 2007a; 2007b). In many regions of the world, including the parts of India, Pakistan, United States, Australia and Mexico, wheat crop is exposed to high temperatures during grain filling period and thus adversely affecting the plant growth, yield and grain quality. High temperature shortens the duration of grain fill and decrease the time to apoptosis and harvest maturity (Altenbach et al., 2003). The exposure to high temperature (>35^0C) at late growth stages is a problem in 40% of the wheat growing areas in the temperate environments. Several approaches based on breeding have been proposed for yield and yield related traits and continue to be important to measure the success of a genotype in heat stressed environments. Every degree rise in temperature above 15^0C exhibits a 3% reduction in yield (Wardlaw et al., 1989). The optimum kernel weight maintained under stress is a good criterion for measure of heat tolerance (Tyagi et al., 2003). Tolerance to heat stress is complex phenomenon and controlled by multiple genes imparting a number of physiological and biochemical changes and no single trait explains the mechanism of heat tolerance. The elucidation of molecular and genetic basis of heat tolerance to identify molecular markers will enhance the efficiency of wheat improvement programmes targeted to develop heat tolerant cultivars. There are two approaches for genetic dissections of complex and quantitative traits; genome-wide scanning and candidate gene approach. Genome-wide scanning is an expensive and resource intensive approach which locates the chromosomal regions of quantitative trait loci at cM level with the aid of molecular markers under population based experimental designs, which usually embed a large number of putative genes. In comparison, the candidate gene approach is more effective and economical method to study the genetic architecture of complex traits (Zhu and Zhao, 2007). Heat shock proteins (HSPs) function as molecular chaperones which are responsible for protein folding, assembly, translocation and degradation in many cellular processes, stabilization of proteins and membranes, and assist in protein refolding under stress conditions including high temperatures (Wang et al., 2004). Proteome studies showed that heat tolerant wheat cultivar exhibited a strong and diverse response to heat stress in the form of HSPs compared to susceptible cultivar (Skylas et al., 2002). Protein profiling after heat stress revealed not only quantitative differences in individual HSPs but also some unique HSPs were found in heat tolerant genotype. Messenger RNA encoding a major class of low molecular weight HSPs (HSP16.9) were detected in wheat genotypes exposed to high temperature (Nguyen et al., 1994). Several reports have shown that different types of HSP are synthesized in different tissues in wheat in response to duration and kind of heat stress (Zivy, 1987; Weng and Nguyen, 1992; Treglia et al., 1999; Rampino et al., 2009; Sharma-Natu et al., 2010; Xu et al., 2011). Most of the studies on HSP in relation to heat stress were based on expression analysis of HSP and but we have not come across any literature on HSP based breeder friendly markers for heat

Table 1. The details of genotypes included for study.

Genotype	Pedigree	Area of adoption	PCR*	SNP (A/G)
AKW 1071	VEE'S'/3/FLN/ACC//ANA	CZ (TS, IR)	+	A
DBW 14	RAJ 3765/PBW 343	NEPZ (LS,IR)	+	A
DBW 17	CMH 79 A. 95 / 3* CNO 79 // RAJ 3777	NWPZ (TS, IR)	+	A
HD 2329	HD 1962/E 4807/3/ K 65/3/HD 1553/UP 262	NWPZ (TS,IR)	-	G
HD 2687	CPAN 2009/HD 2329	NWPZ (TS,IR)	+	A
HD 2733	ATTILA/3/TUI/CARC//CHEN/CHTO/4/ATTILA	NEPZ (TS,IR)	+	A
HD 2833	PBW 226/HW 1042//2285	PZ (LS,IR)	+	A
HS 277	KAVKAZ/CIGUENA	NHZ (TS,RF)	-	G
HUW 510	HD 2278/HUW 234//DL 230 -16	PZ (TS,IR)	+	A
K 7903	HD 1982 / K 816	U.P. (VLS, IR)	-	G
K9107	K 8101/ K 68	NEPZ (TS,IR)	-	G
K 9465	B 1153 / CB 85.HD 2402 / CPAN1830// VEE'S'	NEPZ (LS,RF)	-	G
K 9644	HD 2402 / K 8305	PZ (TS,RF)	+	A
NIAW 34	CNO 79 / PRL "S"	MAH (LS/VLS,IR)	+	A
NW 1014	HAHN'S'	NEPZ (LS,IR)	-	G
PBW 175	HD 2160 / WG 1025	NWPZ (TS,RF)	+	A
PBW 502	W 485/ PBW 343// RAJ 1482	NWPZ (TS,IR)	-	G
RAJ 3765	HD 2402 / VL 639	NWPZ/NEPZ (LS/VLS,IR)	+	A
RAJ 4014	DL 8025/K 9011	RAJASTHAN (TS/IR)	+	A
RAJ 4083	PBW 343 / UP 2442 // WR 258 / UP 2425	PZ (TS,IR)	+	A
UP 2425	HD 2320 / UP 2263	NWPZ (TS,IR)	+	A
VL 616	SKA / CPAN 1507	NHZ (ES,RF)	+	A
VL 804	CPAN 3018 / PAN 3004 // PBW 65	NHZ (TS,IR/RF)	+	A
WH 147	E 4870 / C 286 / C 273 / 4 / S 339 / PV 18	CZ (TS,IR)	+	A
WH 533	AGATHA / YACORA 70	HR (TS,RF)	+	A
WH 542	JUPATECO/ BLUE JAY // URES	NWPZ (TS,IR)	+	A
WH 730	CPAN 2092 / IMPROVED Lok-1	GERMPLASM LINE	+	A

CZ- Central Zone, NEPZ- North Eastern Plain Zone, NHZ- Northern Hill Zone, NWPZ- North Western Plain Zone, PZ- Peninsular Zone, MAH- Maharastra, U.P- Uttar Pradesh, TS-Timely Sown, LS- Late Sown, VLS- Very Late Sown, IR- Irrigated, RF- Rainfed, HR- Haryana
* (+) Presence of PCR band and (–) Absence of PCR band.

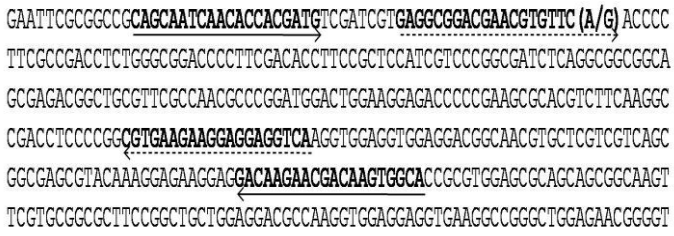


Fig 1. Partial nucleotide sequence of *HSP16.9* gene (GeneBank accession No. X64618). The bold underlined primers were used to amplify the target sequence and the bold dashed primers were used for the amplification of allele-specific DNA fragment.

stress in wheat. Expression analysis also requires high technical skill and it may not be possible to conduct expression studies in segregating populations. In plant genetic research, marker-assisted selection provides a strategy for accelerating the process of wheat breeding. Single nucleotide polymorphisms (SNP) occur in virtually unlimited numbers, and some differences in nucleotide sequences between individuals have potential link with phenotypes which can be converted into genetic markers for high-throughput genotyping (Rafalski, 2002). To the best of our knowledge, there was no previous report in wheat about SNP in HSP associated with thermotolerance. In this study, we have developed a SNP marker in the *HSP16.9* gene of bread wheat to identify heat tolerant and heat susceptible genotypes using an allele-specific PCR primer.

Results and discussion

Heat stress

The average pre-heading maximum and minimum temperatures were almost similar under both trials. During the post heading period the maximum temperature (32.7^0C) was higher by 3.8^0C and minimum (15.3^0C) by 2.5^0C under late sown conditions (Fig. 2a, b).

Phenotypic analysis

ANOVA enables the comparison of means between genotypes. A *P* value of 0.05 or less was considered to be statistically significant. The mean values of grain filling duration (GFD), grain weight per spike (GW), thousand grain weight (TGW), yield (YLD) under heat stress (late sown) (35.4 ± 0.77 days), (1.54 ± 0.21 g), (37.4 ± 2.76 g) and (242.6 ± 41.4) respectively, were significantly lower than that under normal (timely sown) conditions, indicating significant influence of heat stress (late planting) on these traits. Analysis of variance also revealed that genotypes differ significantly for phenological traits, GW, TGW and GGR (Table 2). All these traits suffered reduction under late planting. The average reduction was 12% in GFD, 5% in GW, 10% in TGW and 19% in YLD. The linear regression of reduction in grain weight was determined on other traits. The R^2 value was maximum for TGW (39%) followed by GFD

(11%) and YLD (0.8%). The genotypes differed in extent of reduction for different traits. The grain weight/spike ranged from 1.08g in VL 616 (late sown) to 2.13g in K 9644 (timely sown). During present study, the reduction in grain weight ranged from -24% to 29 %. High temperature reduces grain weight (Wardlaw et al., 1980; Johnson and Kanemasu, 1983), due to reduction in both the duration and rate of grain filling (Sofield et al., 1977; Wardlaw et al., 1980). The high temperature reduces grain weight due to high respiration rate led forced grain development (Tashiro and Wardlaw, 1990; Warrington et al., 1977). Genotypes differ in grain weight in response to high temperature stress after anthesis (Ahmad et al., 1989). Tahir and Nakata (2005) also reported 20% to 40% reduction in main stem grain weight of 18 wheat genotypes. The heat susceptibility index for grain weight/spike was less than 1 in genotypes K 9465, NW 1014, HD 2329, RAJ 4083, VL 804, K 7903, PBW 502, WH 542, UP 2425, DBW 14, HS 277, HUW 510 and DBW 17. Hence these are considered as tolerant for the trait under heat stress. Of these genotypes, HD 2329, RAJ 4083, K 7903, PBW 502, HS 277 and NW 1014 registered significant reduction in grain filling duration.

SNP analysis

A primer set was designed to amplify 307 bp of DNA fragment from HSP 16.9 gene and sequenced from a heat tolerant wheat genotype (K7903) and heat susceptible genotype (RAJ4014). The alignment of both the sequences revealed a base transition from A (heat susceptible genotype) to G (heat tolerant genotype) (Fig.1). The allele-specific primers were designed from the position of base transition. The rationale in designing the primers was based on the premise that the 3'-terminal positions ought to be unique among the known wheat genomic sequences. The analysis of amino acid sequence showed that the base transition positioned 31 resulted in missense mutation from negatively charged aspartic acid to neutral aspargine residue (Asp>Asn). The allele specific marker was used for amplification of DNA fragment in 27 wheat genotypes. Twenty genotypes resulted in presence of band of 197 bp fragment (Table 1 and Fig. 4) and remaining seven genotypes resulted in negative band amplification.

SNP marker vs. terminal heat stress

The phenotypic and genotypic correlation was carried out by single-marker linear regression approach in order to confirm the association between the SNP based marker and the phenotypic traits GFD, GN, GW, TGW and YLD. The adjusted R^2 values of the traits GFD, GN, GW, TGW, and YLD are 5.03%, 9.50%, 29.89%, 24.14% and 8.78%, respectively on the basis of reduction percent. The highest correlation was observed between SNP and GW (Fig 3). Six of the seven genotypes, which resulted into no PCR product, were tolerant for grain weight while all these were tolerant for thousand grain weight. Five of these genotypes suffered significant reduction in grain filling duration. Of remaining 20 genotypes, which resulted into PCR product, only one genotype RAJ 4083 was categorized as tolerant. For further validation of SNP marker, we used a set of eighteen genotypes which were phenotyped for terminal heat tolerance under some other studies. On the basis of heat sensitivity index (HSI) for grain yield, twelve of these genotypes were categorized as tolerant and remaining as susceptible (Table 3). The SNP marker identified ten of the twelve tolerant genotypes. The magnitude of the marker-associated phenotypic effect was 26.63% in the genotypes used for validation. The QTL explaining 12% to 21% phenotypic variation for kernel weight of main spike has also been reported in biparental mapping populations (Mason et al., 2010; 2011). Expression patterns in heat shock protein genes have been linked with adaptation to thermal environments across a range of organisms (Hoffmann and Willi, 2008). Seven proteins were specifically expressed in heat tolerant wheat cultivar Fang but not in heat susceptible cultivar Wyuna after heat shock treatment. Characterization of seven proteins by tandem mass spectrometry revealed five different isoforms of 16.9 kDa HSP (Skylas et al., 2002). Rice (*Oryza sativa*) class I low-molecular mass (LMM) HSP, Oshsp16.9, has been shown to be able to confer thermotolerance in *Escherichia coli*. The deletion amino acid residues in N-terminal domain of Oshsp16.9 led to the loss of chaperone activities (Yeh et al., 2002). The validated SNP of 16.9 kDa HSP may serve as an informative molecular marker that can be used to improve thermotolerance in wheat.

Materials and methods

Plant materials

The plant material comprised of twenty seven genotypes including advanced breeding lines and cultivars of wheat (*Triticum aestivum*) developed at various centres under All India Coordinated Wheat Improvement Program for different agro climatic conditions (Table 1). Field trials were conducted during 2009 - 10 under normal sowing in the month of November (timely) and late sowing in December at Directorate of Wheat Research, (ICAR), Karnal, (Haryana), India. The experiment was laid out in randomized complete block design (RCBD) with two replications. The plot area was 1.2 m^2 and seed rate was 100 Kg/ha. Irrigation was applied as per required while fertilizer application were followed as per recommended agronomic packages and practises. Daily mean maximum and mean minimum temperatures were recorded. Mean minimum and maximum temperatures before and after heading were calculated by taking into consideration the minimum number of days to heading and maximum number of days to maturity.

Phenotypic observation

The observations were recorded for phenology and yield related traits of all the genotypes. The investigated traits were days to heading (DH), days to anthesis (DA), days to maturity (DM), number of grains per spike (GN), grain growth rate (GGR), GW, TGW, GFD and YLD. Phenological traits were recorded at 75% condition. Grain filling duration was calculated as the period from days to anthesis to days to physiological maturity. The five main shoot spikes sampled from each plot were hand threshed to obtain grain number and grain weight/spike. The mean values of these traits under optimum and heat stress conditions were used to estimate the heat susceptibility index (HSI) and calculated by the method suggested by Fischer and Maurer (1978) with the following formula:

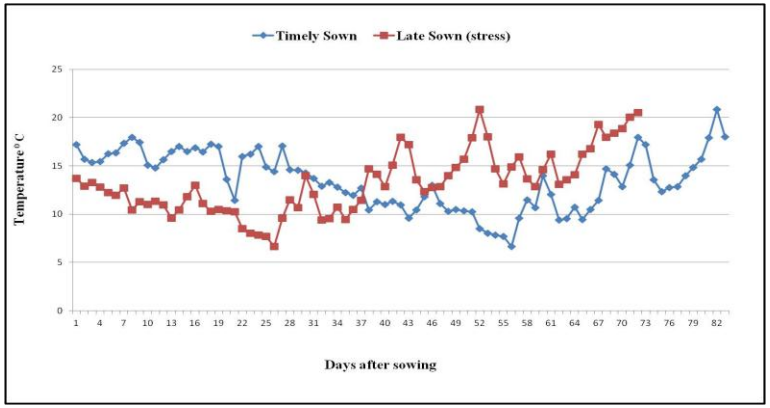
$$HSI = (1 - Xh/X)/(1 - Yh/Y),$$

where Xh and X are the phenotypic means for each genotype under heat stressed and control conditions, respectively, and Yh and Y are the phenotypic means for all genotypes under heat stressed and control conditions, respectively. All the recorded data was subjected to statistical analysis using SAS computer software.

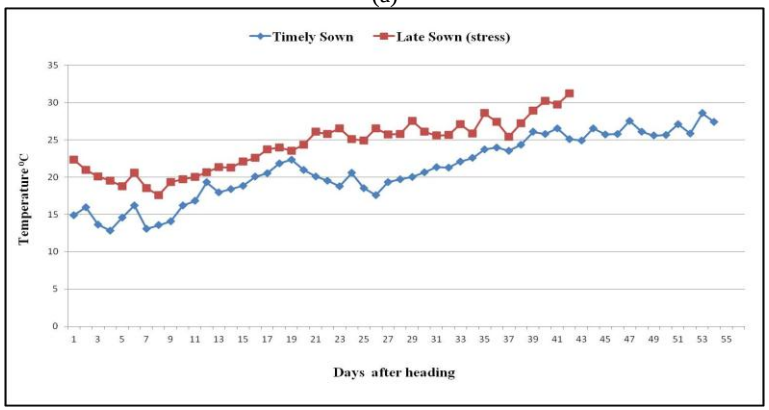
Table 2. Analysis of variance for different traits.

Source of variation	DF	DH	DA	DM	YLD	GN	GW	TGW	GFD	GGR
Genotype	26	66.88**	50.89**	9.28**	5759.58	104.52	0.195*	40.38**	49.49**	0.043**
Condition	1	9501.57**	11367.3**	15265.3**	136960**	102.41	0.17	433.69**	680.01**	0.02
Genotype * Condition	26	13.56*	14.24**	5.81**	6782.24*	17.65	0.04	10.57	8.34	0.02
Residual	27	6.82	1.94	0.46	3377.94	63.10	0.10	7.58	8.06	0.01
Total (corrected)	107	111.19	123.03	146.52	6548.27	59.26	0.10	24.19	23.56	0.02

* $P \leq 0.05$, ** $P \leq 0.01$



(a)



(b)

Fig 2. Daily mean temperature in timely and late sown condition. (a) Line graphs indicate the daily mean temperature during the period from sowing to initiation of heading. (b) Line graphs indicate the daily mean temperature during heading to physiological maturity period.

Table 3. Genotype selected for SNP validation.

Genotype	Heat Susceptibility Index	PCR*	SNP (A/G)
DBW 46	1.34	+	A
HD 2864	0.5	-	G
HD 2932	1.02	-	G
HD 2997	1.12	-	G
HI 1563	0.52	-	G
IC 28913	0.88	-	G
IC 29007A	0.13	-	G
IC 321889	0.8	-	G
IC 321941	0.8	-	G
IC 35117	0.53	-	G
IC 55701A	2.6	+	A
KLP 1045	0.85	+	A
MP 4010	1.12	+	A
NEPAL 8	0.6	+	A
PBW 621	0.72	-	G
PDW 315	1.14	-	G
RAJ 4258	0.7	-	G
UAS 316	1.23	+	A

* (+) Presence of PCR band and (–) Absence of PCR band

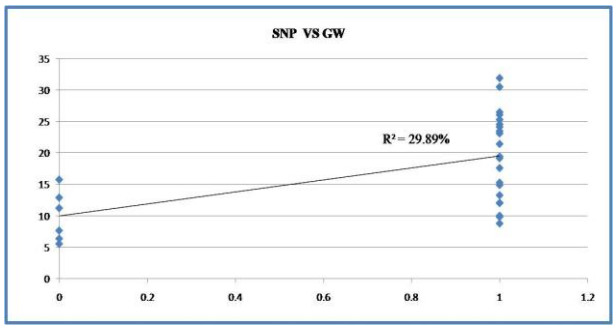


Fig 3. Correlation of SNP Marker with grain weight/ spike (GW).

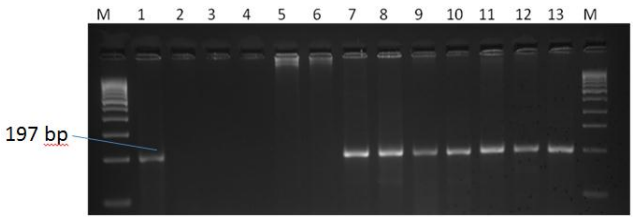


Fig 4. PCR analysis using the SNP marker Lanes: Lane 2-6 Heat tolerant genotypes; 2- K 7903, 3- PBW 502, 4- K 9107, 5- HD 2329, 6-K 9465, and Lane 1, 7-13 Heat susceptible genotypes; 7- WH 147, 8- PBW 175, 9-WH 542, 10- HUW 510 , 11- HD 2733, 12- HD 2687, 13-VL 616, M. DNA ladder 100bp.

DNA extraction and PCR analysis

Total genomic DNA was isolated from leaves of 3 week old seedlings by CTAB method (Doyle and Doyle, 1990) of all the genotypes. A primer set was designed to amplify the region consisting of nucleotides 14–320 (307 bp) of the bread wheat *HSP16.9* gene (GenBank accession No. X64618). The primers used for the polymerase chain reactions (PCRs) were 5′-CAGCAATCAACACCACGATG-3′ (forward) and 5′-TGCCACTTGTCGTTCTTGTC -3′ (reverse), which are shown in Fig. 1. For marker analysis, a polymerase chain reaction (PCR) was performed in a 25 µl volume containing 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 200 µM of each dNTP, 0.2 µM of each primer and 1.0 unit of *Taq* DNA polymerase (Bangalore Genei, India). The thermocycling program consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of 45 sec at 94°C, 45 sec at 60°C, 60 sec at 72°C and a final cycle of 5 min at 72°C in S 1000 Thermal Cycler (Bio-Rad). The amplified product were separated by electrophoresis on 3% (w/v) agarose gel and stained with ethidium bromide and analyzed under UV light.

SNP detection and designing of allele specific primer

The PCR products were purified using HiPurA^{TM} agarose gel DNA purification spin kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India) according to manufacturer's instructions. The PCR products were sequenced from heat tolerant wheat genotype (K7903) and heat susceptible genotype (RAJ4014) from both the directions to minimize the false SNP due to sequencing artefacts. The sequencing was performed by Eurofins Genomics India Pvt. Ltd, Banglore, India). The sequence alignments were done using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default parameters. After sequence alignment, forward primer was designed by selecting primer ending with a mismatch (SNP) at the 3′ terminus. Primers used in this study were designed using the software Primer3 (Rozen and Skaletsky, 2000).

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