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Development and validation of heat-responsive candidate gene and miRNA gene based SSR markers to analysis genetic diversity in wheat for heat tolerance breeding

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Abstract
Being a major staple food crop of the world, wheat provides nutritional food security to the global populations. Heat stress is a major abiotic stress that adversely affects wheat production throughout the world including Indo-Gangatic Plains (IGP) where four wheat growing countries viz., India, Bangladesh, Nepal and Pakistan produce 42% of the total wheat production. Therefore, identification of heat stress responsive molecular markers is imperative to marker assisted breeding programs. Information about trait specific gene based SSRs is available but there is lack of information on SSRs from non-coding regions. In the present study, we developed 177 heat-responsive gene-based SSRs (cg-SSR) and MIR gene-based SSR (miRNA-SSR) markers from wheat genome for assessing genetic diversity analysis of thirty- six contrasting wheat genotypes for heat tolerance. Of the 177 SSR loci, 144 yielded unambiguous and repeatable amplicons, however, thirty-seven were found polymorphic among the 36 wheat genotypes. The polymorphism information content (PIC) of primers used in this study ranged from 0.03–0.73, with a mean of 0.35. Number of alleles produced per primer varied from 2 to 6, with a mean of 2.58. The UPGMA dendrogram analysis grouped all wheat genotypes into four clusters. The markers developed in this study has potential application in the MAS based breeding programs for developing heat tolerant wheat cultivars and genetic diversity analysis of wheat germplasm. Identification of noncoding region based SSRs will be fruitful for identification of trait specific wheat germplasm.

Keywords Cg-SSRs · miR-SSR · Genetic diversity · Wheat · Heat tolerance · Heat susceptibility index

Introduction

Wheat is an important staple food for human consumption around the world. Major population expansion is predicted to occur in Asian countries, particularly China and India which are already most populous countries in the world. These countries rely on wheat for their calorific demands.

To meet out the food demand of the increasing population, wheat production also needs to be increased upto 40–50% by the end of 2050. Heat stress adversely affects the wheat production and quality all over the world including India. Different wheat growing regions in India, encounter heat stress at different wheat growth stages for instance the peninsular and central parts came across heat stress during the crop season (early heat), while north-western plains face terminal heat. Farmers in north -west and central India takes advantage of residual moisture in soil for sowing of wheat to develop wheat genotypes for both early and terminal heat tolerance. High temperature (> 30 °C) at succeeding stages of wheat has profound impact on grain filling [1–4] and anthesis [5, 6]. Wardlaw et al. [5] shown that the grain yield of wheat per spike was reduced by upto 4% per 1 °C rise in temperature above 15 °C under controlled experiments. Studies have reported less damage to grain contents when daily temperature rises gradually

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in comparison to sudden rise at grain filling stages [7]. Increasing temperature affects a large proportion of wheat growing areas as revealed by current trends about global warming. The report submitted by IPCC [2014] for a period of 1880–2012 indicates that the average global temperature has increased by 0.85 °C. Despite its importance, the genetic basis of heat stress tolerance is poorly understood in wheat. In this perspective, there is ardent need for raising heat tolerant wheat varieties capable of mitigating heat stress by utilizing novel genetic resources. In the past, studies have been conducted to use genetic variation in the form of QTLs among existing wheat cultivars for abiotic stress tolerance [8–10]. SSR markers show preferred advantage of co-dominance, locus specificity and high polymorphism and therefore have been utilized for evaluating genetic variation within the population [11–15]. SSRs are preferable over SNPs because of their multi-allelic nature [16] and have been widely distributed in the coding and non-coding genomic regions of plant genome [17, 18].

SSRs can be developed from either functional regions (genic-SSR) or random sequences of the genome [19, 20]. SSRs developed from functional regions are being effectively utilised in molecular breeding programs such as markers assisted selection, QTL tagging and association mapping [21]. Association of heat stress-responsive SSRs in diverse accessions were previously shown by Sun et al. [22]. Similarly, Kumar et al. [23] reported genic-SSR markers in wheat, while miRNA- based markers in Brassica [24], rice [25] and bean [26]. As per published data, only a few studies exist on utilization of SSRs from non-coding regions [27]. A number of SSR markers are currently available in various plant species, identification of trait specific SSR markers remains a daunting task in case of polyploidy crops such as wheat.

In this study, we mined heat stress-responsive genes/ miRNA from wheat genome and developed SSRs. The genetic diversity of 36 wheat genotypes with contrasting heat stress tolerance was assessed using thirty-seven polymorphic heat-stress responsive genes based and miRNA gene based SSR markers. The wheat genotypes were phenotyped at ICAR-Indian Institute of Wheat and Barley Research (IIWBR), Karnal under normal sown and later sown field conditions (29°42′N, 77°2′E) for assessing heat susceptibility index. The data obtained from this study contribute to a better understanding of the genetic diversity in wheat, and will provide the basis for further dissecting the function of genetic regions during plant growth and development in response to heat stress. This study is the first report on development and validation of heat-responsive cg-SSR and miR-SSRs in wheat. The information generated in this study will be valuable for the marker-assisted breeding programs targeted heat tolerance in wheat.

Materials and methods

Plant material, phenotyping and data recording

The experimental material comprised of 36 diverse wheat genotypes consisting heat susceptible and heat tolerant wheat genotypes. These were phenotyped at the IIWBR, Karnal, India (29°42′N, 77°2′E) for two consecutive crop seasons 2016–17 and 2017–18 under two non-stress (November19, timely sown) and stress conditions (December19, late sown) with two replications. Each replication comprised of square shaped (0.5 m L × 0.5 m W) plots and each genotype occupied single plot for precise phenotyping under field condition. Each plot had four rows with six plants per row. Row to row spacing and plant to plant spacing distance was 10 cm. To ensure precision in planting, a dibbling tool, named *Dibbler*, was fabricated [13]. For recording the data, inner eight plants from 2nd and 3rd rows were utilized to avoid border effects and followed standard agronomic practices. Data were recorded for traits like days to heading (DH), days to anthesis (DA), days to maturity (DM), Grain filling duration (GFD), productive tillers (PTL), thousand grain weight (TGW) and GY (grain yield), BM (Biomass) and CTD (canopy temperature depression). The details of the wheat genotypes used in this study were given in Table 1. In order to assess the effect of heat stress, the heat susceptibility index (HSI) was calculated using the following equation.

$$HSI = [1 - (Y_s/Y_t)/(1 - X_s/X_t)]$$

where Y_s is the grain yield under stress, Y_t is the grain yield under non-stress, X_s and X_t are the mean yields of all genotypes under stress and non-stress conditions, respectively [28].

Development of heat -responsive candidate gene and miRNA based SSRs

We have selected functionally characterised heat-stress responsive candidate genes from wheat and other model plant species [29–31]. The sequence information of the identified genes was retrieved from the ensemble plant genome database. These protein/gene sequences were used as query in Ensembl Plants wheat genome database to identify the respective wheat orthologs. BlastPrimer3 online database was used with default parameters for mining and designing of genomic SSRs as listed in Supplementary Table 1.

DNA extraction and PCR amplification

Two week old wheat seedling young leaves were collected from 36 wheat genotypes and genomic DNA was isolated

Table 1 Evaluation of heat susceptibility index for grain yield

Sr No	Genotypes	Pedigree	HSI (GY)	Tolerance*
	HS240	AU/KAL-BB//WOP"S"/PAVON"S"	1.32	LHT
	HUW468	CPAN-1962/TONI//LIRA"S" / PRL'S'	0.77	MHT
	HUW510	HD 2278/HUW 234//DL 230-16	1.02	LHT
	PBW343	ND/VG1944//KAL//BB/3/YACO'S'/4/VEE#5'S'	1.08	LHT
	PBW550	WH594/RAJ3856/W485	1.80	LHT
	C306	RGN/CSK3//2*C591/3/C217/N14//C281	1.23	LHT
	DBW14	RAJ3765/PBW343	0.49	HHT
	DBW17	CMH79A.95/3*CNO79//RAJ3777	1.12	LHT
	GW322	GW 173/GW 196	0.10	HHT
	HI1500	HW2002*2//STREMPALLI/PNC5	0.63	MHT
	NI5439	REMP 80/3* NP 710	1.34	LHT
	NIAW34	CNO79/PRL"S"	1.27	LHT
	NW1014	CEBECO-148/KATHADIN/7/HEINES-KOLBE N/MIDA/4/4777/3/REI//YAQUI/KEN-TANA-48/5/YECORA-70/6/TUCAN	1.31	LHT
	RAJ3765	HD2402/VL639	0.81	HHT
	UP2338	UP 368/VL 421//UP 262	1.04	LHT
	VL616	SONALIKA/CPAN 1507	1.23	LHT
	WH1080	21STSAWSN151	0.91	MHT
	WH147	E 4870/C286/C273/4/S339/PV18	0.92	MHT
	CBW38	CNDO/R 143/ENTE/MEXI_2/3/Ae.SQUARROSA(TAUS)/ 4/WEAVER/ 5/2*PASTOR	0.88	MHT
	DBW90	HUW468/WH730	0.98	MHT
	HD2687	Gene introgressed from Sr31 Lr26 Yr9 Pm8	0.76	MHT
	HD2967	ALD/CUC//URES/HD216OM/HD2278	0.78	MHT
	HD2808	WH 542/DL 377–8	0.32	HHT
	HD2932	KAUZ/STAR//HD 2643	1.28	LHT
	K7903	HD 1982/K816	0.96	MHT
	MACS6145	C306 + LR28	0.76	MHT
	MACS2496	SELECTION FROM SERI 'S'	0.88	MHT
	MP4010	ANGOSTURA 88	1.06	LHT
	PBW590	WH 594/RAJ3814//W 485	1.06	LHT
	UP2382	CPAN 2004/HD 2204	0.85	MHT
	WH730	CPAN 2092/Improved Lok-1	1.34	LHT
	WR544	KALYANSONA/HD1999//HD2204/DW38	1.06	LHT
	RAJ4037	DL788-2/RAJ3717	0.37	HHT
	RAJ4079	UP 2363/WH 595	0.93	MHT
	RAJ4083	PBW 343/UP 2442//WR 258/UP 2425	0.71	MHT
	RAJ4210	HW 2042/Lok-1 412w/xV/HW 2042/Lok-1	0.87	MHT

HHT designated by an HSI value less than 0.50; MHT with values between 0.51–1.0 and LHT with HSI value more than 1.1

*HHT- Highly heat tolerant; MHT- Moderatly heat tolerant; LHT- Low heat tolerant; GY- Grain yeild

following CTAB method [32], quantified by UV_{260} absorbance and adjusted to 25 ng/µl. All 177 SSR primers were used for marker analysis. The PCR volume was 20 µl, consisting of 13.375 µl of nuclease free water, 0.5 µl of dNTPs, 2.5 µl of 10×buffer, 1.5 µl of $MgCl_2$, 1 µl of each of forward and reverse primers and 0.125 µl of *Taq* DNA polymerase and 1 µl of template DNA. The thermal cycling consisted of 94 °C for 3 min (initial denaturation); 35 cycles of 94 °C for 1 min., 55 °C for 1 min the annealing temperature, and 72 °C for 1 min; followed by a final extension of 72 °C for 10 min. PCR products were resolved in a vertical 6% PAGE system at 140 V and visualized under UV light gel documentation system (Alpha Innotech, USA).

SSR marker scoring and statistical analysis of genetic diversity

Scoring of amplified fragments was done as 1 for presence of band and absence of band was scored as 0. The data was entered as discrete variable in the binary matrix. We used POPGENE version 1.31 [33] to find *Ne* (total number of effective alleles), *Na* (total number of alleles) and Shannon's Information index (*I*). Power-Marker version 3.25 was used to calculate Polymorphic Information Content (PIC) for each SSR marker loci [34]. The phylogenetic relationship among the wheat genotypes was drawn using DARwin version 6.0.9 [35]. Phylogenetic tree was constructed using the unweighted neighbour-joining method followed by bootstrap analysis with 1000 permutations. To decipher the level of genetic variations among the genotypes and within the populations molecular variance (AMOVA) analysis was executed. The genetic distance matrix was utilized for showing AMOVA study. The number of sub-populations determined based on STRUCTURE results were used for AMOVA. The AMOVA was performed using software GenAlEx6.5 [36].

Population structure analysis

The population structure was assessed in a model-based approach using the STRUCTURE software ver 2.3.4 [37], the run length was set to 150,000 MCMC (Markov chain Monte Carlo) using correlated allele frequencies under an admixture model. Structure analysis were carried out by setting the number of sub-populations (k) from k = 1 to k = 10. The optimum k value was determined based on an adhoc statistic Δ*k* [38] and the rate of change in the log probability of data between successive k values.

Results

Phenotypic analysis for assessing terminal heat tolerance in wheat genotypes

The average minimum and maximum temperature under normal sown conditions was 11.46 °C and 27.10 °C (2016–2017; Fig. 1a) and 11.40 °C and 25.60 °C (2017–2018; Fig. 1b), respectively. HSI was calculated to reflect the performance of genotypes under heat stress environment. HSI < 1 indicates the tolerance of genotype to heat stress, whereas HSI > 1 indicates susceptibility of the genotypes under stress. A comparative analysis of HSI was used to characterize genotypes with least susceptibility to thermal stress. HSI for grain yield in the present study ranged from 0.10 to 1.80 and 19 genotypes possessing

HSI < 1 (HUW 468, DBW14, GW322, HI1500, RAJ3765, WH1080, WH147, CBW38, DBW90, HD2687, HD2967, HD2808, K7903, MACS2496, UP2382, RAJ4037, RAJ4079, RAJ4083, and RAJ4210) were categorised as heat tolerant (Table 1). The data recorded on the morpho-physiological components viz. DTH, DTA, DTM and GFD under both conditions for HHT ranged from 90.38–84.04, 102–97.1, 135.8–121.3 and 64.2–122.9 respectively. However, mean value for MHT based on the agronomical traits are 91.7–82.7, 103.0–93.0, 136.9–122.2 and 36.2–29.1, respectively (Supplementary Table 2). The average value for above mentioned components for LHT genotype traits is 92.3–83.2, 102.7–92.7, 138.3–122.5 and 37.7–29.8, respectively. The pooled HSI for other traits ranged from 0.10 to 2.66 for thousand grain weight, 0.94 to −3.16 for biomass, and −1.84 to –2.26 for cell membrane stability. HSI as an indicator of stability for heat tolerance has already been analysed [13]. The various morpho-physiological traits analysed in this study under stressed and non-stressed conditions revealed that GY, BM, TGW and CTDH have a strong correlation (Supplementary Table 3). Pearson correlation coefficient was calculated for all the combinations. BM had positive correlation with GY (0.681) and TKW (0.344) while under stress condition TGW was significantly correlated with CTD (0.342) and GY2 (0.431) as shown in Supplementary Table 4.

Development and validation of heat-responsive cg-SSR and miRNA-SSR markers

To carry out this study, heat stress-responsive genes catalogued in wheat and the wheat orthologos in other crops were utilized for developing heat-responsive cg-SSR and miRNA-SSR [29–31]. The list 157 SSRs designed using 139 heat stress responsive genes including 10 miRNA are presented in Supplementary Table 1. Out of the 157 SSRs, 80 SSRs are located within the exon region, 33 SSR in intron region, 17 SSR located in the 5′ UTR region and rest 16 SSRs located in 3′ UTR (Fig. 2). Most of the identified SSRs were located in transcribed regions of wheat heat stress responsive genes.

Out of 157 SSRs screened, 144 SSRs primer pairs yielded unambiguous and strong amplification. Among these 37 polymorphic SSRs were selected and screened on a panel of 36 wheat genotypes comprising both heat tolerant and heat susceptible for assessing their genetic diversity (Table 2). The analyses revealed a total of 108 polymorphic alleles ranging from 2 to 6 per SSR locus, with an average of 2.76 alleles per locus. However, there are as many as nine SSRs i.e. PSSR-HSP105, PSSR-CBK3, PSSR-UVH6, PSSR-Hsp23.7, PSSR-HsfA7, PSSR-Myb2, PSSR-ACA6, PSSR-miR169m
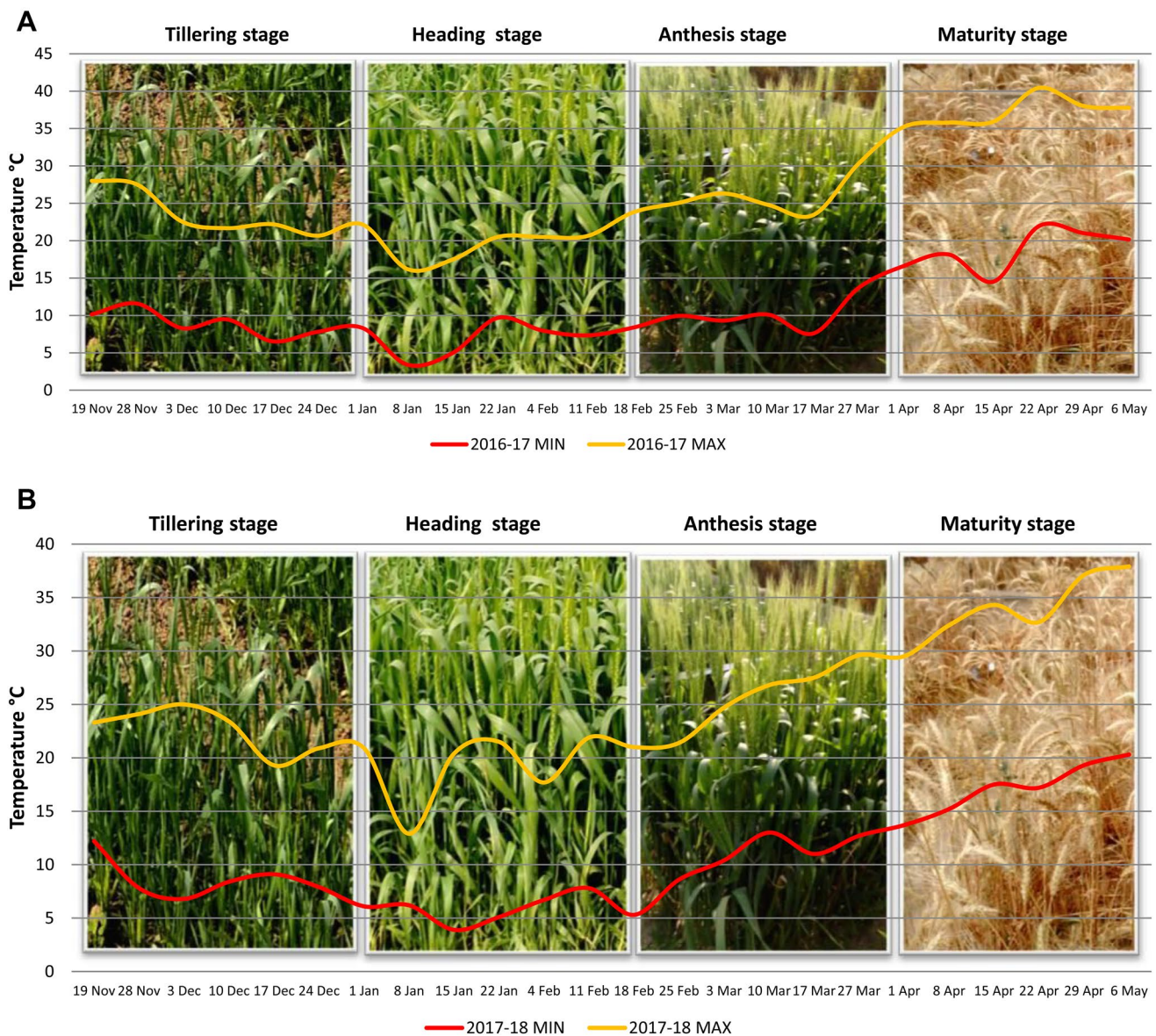
Fig. 1 Graphical representation of daily maximum and minimum temperature under timely and late sown conditions, **a** during crop season 2016–17 with average maximum temperature of 27.10 and average minimum temp of 11.46, **b** during crop season 2017–18with average maximum temperature of 25.60 and average minimum temp of 11.40

and PSSR-miR169n, which yielded more ≥ 4 alleles (Table 2). PSSR-HSP-70 and PSSR-RacB had the lowest PIC values (0.03), and PSSR-GGT has the highest PIC value of 0.73 (Table 2), which indicates their potential use for genetic diversity and genetic mapping analysis. A representing banding pattern of 36 wheat genotypes obtained with PSSR-miR160amarkers showing allelic variation for HHT was shown in Fig. 3.

Population structure and cluster analysis

Population structure analysis grouped the genotypes into four subgroups facilitating identification of group clustering and PCA (principal coordinate analysis) analysis (maximum of the ad hoc measure ΔK showed a clear peak at an optimal K value of four, $K = 4$). The genotypes with the probability of $\geq 80\%$ were assigned to corresponding subgroups and
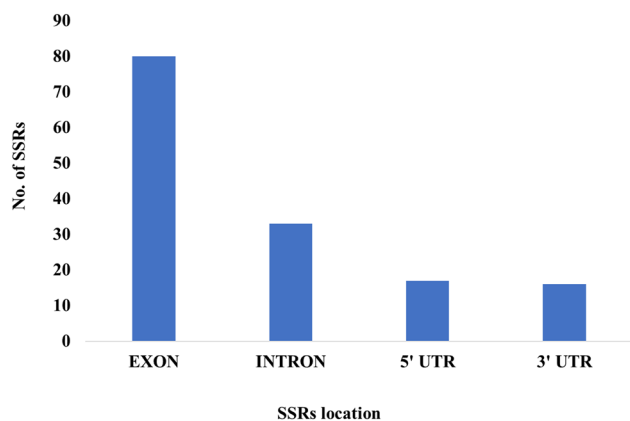
Fig. 2 SSRs distribution in the heat stress-responsive genes

those with ≤ 80% were defined as admixtures [39]. Genotypic data of 37 SSRs on 36 wheat genotypes was analysed using this software Darwin. Structural analysis revealed four sub-populations (Fig. 4b). Also, PCA analysis clustered 36 genotypes into 4 groups as shown in Fig. 4c. The 36 wheat genotypes were found to be genetic diverse from each other among the four populations (Supplementary Table 5).

The relatedness of 36 wheat genotypes at genetic level was determined by nj- analysis based on the 37 polymorphic cg-SSRs and miRNA-SSRs amplification results. The 36 wheat genotypes could be grouped into four major clusters based on the cluster results (Fig. 5). The four groups comprised of 5, 11, 11, 9 and wheat genotypes respectively based on cluster analysis. HHT genotypes clustered separately in group-I (Fig. 5), followed with mixed pattern for rest of the groups. The data obtained from trait specific SSRs in this study might be helpful for better understanding of the genetic diversity in wheat. The principle coordinate analysis demonstrated that the 36 wheat genotypes can be separated distinctly from each other as shown in Fig. 4c. Although all the 37 markers were found polymorphic but only four of the distinguished the wheat genotypes in contrasting groups for heat stress tolerance (Fig. 3).

Differentiation of populations on genetic basis

Information obtained from STRUCTURE analysis was further exploited for AMOVA analysis and the genetic diversity indices using GenAlex 6.5 software. The AMOVA showed that 22.26% of the total variation was found among/between sub-populations, while, the rest of variation (77.74%) was due to individuals/genotypes present within sub-populations (Supplementary Table 6). These findings indicate that genetic differentiation was low among sub-populations and high within subpopulation. The values obtained for four

groups for various genetic parameters have been detailed in Supplementary Table 6.

Discussion

Simple sequence repeats (SSRs) markers are extensively used for molecular analysis of plants due to their high reproducibility, co-dominant, polymorphic, and cross-transferable nature. SSR markers derived from genic regions are valuable genomic resource targeting trait mapping selection in future crop breeding programmes. Marker assisted breeding is a faster way to develop improved varieties for heat stress tolerance which is a practical option for enhancing wheat production for wheat growing in warmer areas. Genic SSRs located in the coding region of the genome is relatively easy and inexpensive, and highly transferable to related taxa [40]. Because of these advantages, genic SSRs have been developed and used in many plant species. SSRs with in the miRNA precursors have been identified and used for diversity analysis. Previous studies showed in silico identification of SSR markers derived from genes involved in zinc and iron transporter [41], rice [19]; foxtail [42] or miRNA based SSRs in *Cleistogenes songorica* [43], tea [44], lotus [45] and *Punica granatum* [46]. In this study, we developed microsatellite loci from heat stress-responsive candidate genes and non-coding regions (miRNA) using publically available and validated genes for heat/abiotic stress tolerance in wheat [29–31]. These markers were utilised in characterizing genetic variation and population structure analysis in a panel of diverse wheat germplasm representing heat tolerant and susceptible genotypes. The present study is the first report of cg-SSR and miRNA-SSR marker development in wheat for heat stress tolerance.

Genic SSRs have potential to perform as functional markers and they have more advantages over non-genic SSRs [47–49]. Bej and Basak [50] reviewed extensively the role of miRNAs as potential biomarkers in plants under different stresses. One of the benefits of using miRNA-based markers is that it is not necessary to work out Marker traits association (MTAs) for these markers using QTL analysis, because their association with specific traits is already known in most cases. Thus, in our study out of 157 SSRs, 80 SSRs are located in the exon region, whereas, 17, 16 and 33 SSRs were located in the 5′ UTR, 3′ UTR and intronic regions, respectively and 11 SSRs were derived from the miRNAs (Supplementary Table 1). Genetic diversity and PIC are extremely helpful for studying the level of polymorphisms among genotypes in plant breeding programs. The PIC values can be classified in to three categories as reported by Botstein et al. [51] that if the PIC value of the

Table 2 Polymorphic gene/miRNA based-SSR markers in this study for heat tolerance in wheat

Sr no	Gene name	Gene function	Primer name	SSR location	Primer sequence	No. of allele	PIC value	Allele size	GD
	TaHSP22.0-ER	Heat shock protein	PSSR-TaHSP57	3′ UTR	F 5′-GTAGGAGAGACCACGACGAGAG-3′ R 5′-AACTGAACAAGACACGACAGC-3′	2	0.21	180–340	0.24
	TaACD58.7- NaLi	Heat shock protein	PSSR-TaHSP61	5′ UTR	F 5′-TTAACTGATGCTCGTGGAGAG-3′ R 5′-CATGGAGAGGAAGGTGGAG-3′	2	0.36	100–300	0.48
	TaACD16.3- CIX	Heat shock protein	PSSR-TaHSP70	Exon	F 5′-ATTTATCCTGGGGTCGTCA-3′ R 5′-CTCCGGGCAACAGTCTTC-3′	2	0.03	110–210	0.02
	TaACD22.4-CVII	Heat shock protein	PSSR-TaHSP81	3′ UTR	F 5′-GAATGAGGCAAGCAATACACA-3′ R 5′-AGCCAATAAAGTGGGACCAT-3′	2	0.34	190–230	0.43
	TaHSP23.0B-MTI	Heat shock protein	PSSR-TaHSP94	Exon	F 5′-GTACAACACCCAGGTCAAGG-3′ R 5′-ACGGAAAAAGCTCATCAGAAC-3′	2	0.43	200–270	0.48
	TaHSP19.0-CIII	Heat shock protein	PSSR-TaHSP97	Exon	F 5′-GGACAACGTGCTGGTGAT-3′ R 5′-GACGGACTTGGTCTTCTTCTC-3′	3	0.68	180–500	0.72
	TaHSP27.5-CII	Heat shock protein	PSSR-HSP105	Intron	F 5′-CACTCCTGCCTCCTCCTC-3′ R 5′-GACCAGCTCACCAGCAAGTA-3′	5	0.43	150–300	0.49
	TaHSP22.9-MTI	Heat shock protein	PSSR-HSP107	Exon	F 5′-GTACAACACCAAGGCCAAG-3′ R 5′-GTTGACACTTCAGCACGAAC-3′	3	0.22	190–300	0.26
	TaCHIP	E3 ubiquitin ligase	PSSR-CHIP	5′ UTR	F 5′-CGCAATCCCAACAACTT-3′ R 5′-CCTCGGTGTAGGCAGTCGAT-3′	2	0.69	180–500	0.74
	TaCBK3	Ca2+/calmodulin-binding protein kinase	PSSR-CBK3	3′ UTR	F 5′-GTGGATTGATGATGGTGTGT-3′ R 5′-CACCAAAACAAGGACACTGAC-3′	4	0.34	170–490	0.44
	TaMIPS2	myo-inositol 1-phosphate synthase	PSSR-MIPS2	3′ UTR	F 5′-TGGAGTACAAGTGAGGAGCAG-3′ R 5′-AAAGGAAACCGAAGACAAGC-3′	2	0.37	180–200	0.49
	TaRPN1A	26S proteasome subunit	PSSR-RPN1A	Exon	F 5′GAGTCTGCCTCCGTACGAC-3′ R 5′-CCTTCTTGTCGTCCTTCTTCT-3′	2	0.54	200–500	0.61

Table 2 (continued)

Sr no	Gene name	Gene function	Primer name	SSR location	Primer sequence	No. of allele	PIC value	Allele size	GD
	TaUVH6	DNA repair helicase/	PSSR-UVH6	5′ UTR	F 5′-GAATTCCACCGAACACCTC-3′ R 5′-GAACTTCATGGTGACGGCTA-3′	4	0.07	110–400	0.07
	TaHSFB2b	Heat shock factor	PSSR-HSFB2b	5′ UTR	F 5′-GGAAGGTTGCTTTGACACC-3′ R 5′-CACCAGCTGATACGTCTTGTT-3′	2	0.23	100–200	0.27
	TaHSFB2b-1	Heat shock factor	PSSR-HSFB2b-1	Exon	F 5′-CGTCCTCATGTCCAAGTACAC-3′ R 5′-CATCTTCTCCTCCTCGTCCT-3′	2	0.06	160–190	0.06
	TaHSFC1a	Heat shock factor	PSSR-HSFC1a	Exon	F 5′-CCGTGATTTTACTTCCTTTCC-3′ R 5′-GCTGCTCGAAGGTTGTGA-3′	2	0.37	100–200	0.49
	TaACD20.0B-CII	Heat shock protein	PSSR-HSP20-2	Exon	F 5′-GGAGGCCATCTCACTGACT-3′ R 5′-ACCTCCACCTTCACCTCCT-3′	2	0.61	150–510	0.67
	TaHSP23.7-MTI	Heat shock protein	PSSR-HSP23.7	Exon	F 5′-TACAAACACCCAGGCCAAG-3′ R 5′-ACGTCCTCCATCAGAGACAG-3′	4	0.21	150–300	0.24
	TaGGT	Glycosylation	PSSR-GGT	Intron	F 5′-ACTTCCTCCTACTCCACGTC-3′ R 5′-GATCTCCGACACCCACTT-3′	2	0.73	150–380	0.76
	TaHsfA7	Heat shock factor	PSSR-HsfA7	Exon	F 5′-GGTTTTGGACTTGGTAGAACT-3′ R 5′-CACTAACCCCAAATATCGAA-3′	6	0.07	160–400	0.06
	TaAKT1	Ion transportor	PSSR-AKT1	Intron	F 5′-GTCCAGAACGTCTACCTCTTC-3′ R 5′-GAAACAAGGTAACCAGTTGAG-3′	2	0.10	250–400	0.10
	TaCML11	Ca^{2+}-binding protein	PSSR-CML11	Exon	F 5′-AACAACCTCCAACGCTCT-3′ R 5′-GTCGAAGGACCGGAAGAT-3′	2	0.63	170–370	0.68
	TaMyb2	Transcription factor	PSSR-Myb2	Exon	F 5′-CTAGCTTCGTTTGTCTGT GT-3′ R 5′-GATGTAGTTGGCGAGGAG-3′	4	0.31	130–460	0.39
	TaDREB1F	Transcription factor	PSSR-DREB1F	Exon	F 5′-AAGAGGAGACCGTCAAGA AC-3′ R 5′-CTCGATGTCGATGAGCAG-3′	2	0.44	110–350	0.50
	TaRacB	GTPase	PSSR-RacB	Exon	F 5′-ATGTGAGATGCTGACAGA GTT-3′ R 5′-TAGATCACAAGATGGAGC AAG-3′	3	0.03	150–300	0.03
	TaGR3	Reduces GSSG	PSSR-GR3	Exon	F 5′-CCACACTTCCAGAAAGGA-3′ R 5′-GTGAAGAGGTCGTAGTCG TG-3′	2	0.35	155–360	0.46

Table 2 (continued)

Sr no	Gene name	Gene function	Primer name	SSR location	Primer sequence	No. of allele	PIC value	Allele size	GD
	TaZFP252	Transcription factor	PSSR-ZFP252	Exon	F 5'-AGGAGGAAACGAAGGATG-3' R 5'-CTGAGGGACGGCTCCTGCT-3'	2	0.61	100–220	0.67
	TaACA6	Ca²⁺ Atpase	PSSR-ACA6	Exon	F 5'-ACACACACTCCCATCCT-3' R 5'-GTTCTCCTCCAGGTAGCT CT-3'	4	0.10	140–370	0.11
	TamiR160a	miRNA	PSSR-miR160a	–	F 5'-GAGGTGAAAACAATGGGA TA-3' R 5'-CCAGGAATCTAAAGCAAC C-3'	2	0.66	180–240	0.71
	TamiR169m	miRNA	PSSR-miR169m	–	F 5'-TGCCATGTAGAGAGAGAG AGA-3' R 5'-TATATCCACAGGCAAGTC ATC-3'	6	0.40	150–365	0.51
	TamiR169k	miRNA	PSSR-miR169k	–	F 5'-TGCCATGTAGAGAGAGAG AGA-3' R 5'-TATATCCACAGGCAAGTC ATC-3'	3	0.30	160–270	0.37
	TamiR171a	miRNA	PSSR-miR171a	–	F 5'- GTCCGTCGTAAACCTAAC ATA-3' R 5'- CGACGAGCAGTGAGATAT AAG-3'	2	0.10	170–200	0.11
	TamiR172c	miRNA	PSSR-miR172c	–	F 5'-ATGTATAGGACAAAGGGA AGC-3' R 5'-ATCAAGATTCACATCCAT CC-3'	2	0.32	180–190	0.40
	TamiR10	miRNA	PSSR-miR10	–	F 5'-GAGATTATTGTGGTACGT GGA-3' R 5'-TATTAAGTGCCTCTGCCAGT-3'	2	0.65	150–220	0.71
	TamiR169n	miRNA	PSSR-miR169n	–	F 5'-GTGACTCTTATCGTTCAT GCT-3' R 5'-ACTCCTACAAAACATGCA GAG-3'	4	0.08	170–290	0.08
	TamiR19	miRNA	PSSR-miR19	–	F 5'-TCTCACTAGACCCCTCTC TTC-3' R 5'-GTTTGAGGTGCTACAAAT GG-3'	2	0.48	150–180	0.57
	TamiR172d	miRNA	PSSR-miR172d	–	F 5'-TATTAAGTGCCTCTGCCAGT-3' R 5'-GAGATTATTGTGGTACGT GGA-3'	3	0.40	100–190	0.45
			Average			2.76	0.35		0.45

Fig. 3 A representative amplification profile of the PSSR-miR169m (heat–responsive SSR primer). Image was taken in gel documentation system after staining with ethidium bromide. Arrow shows allele amplified in HHT wheat genotypes
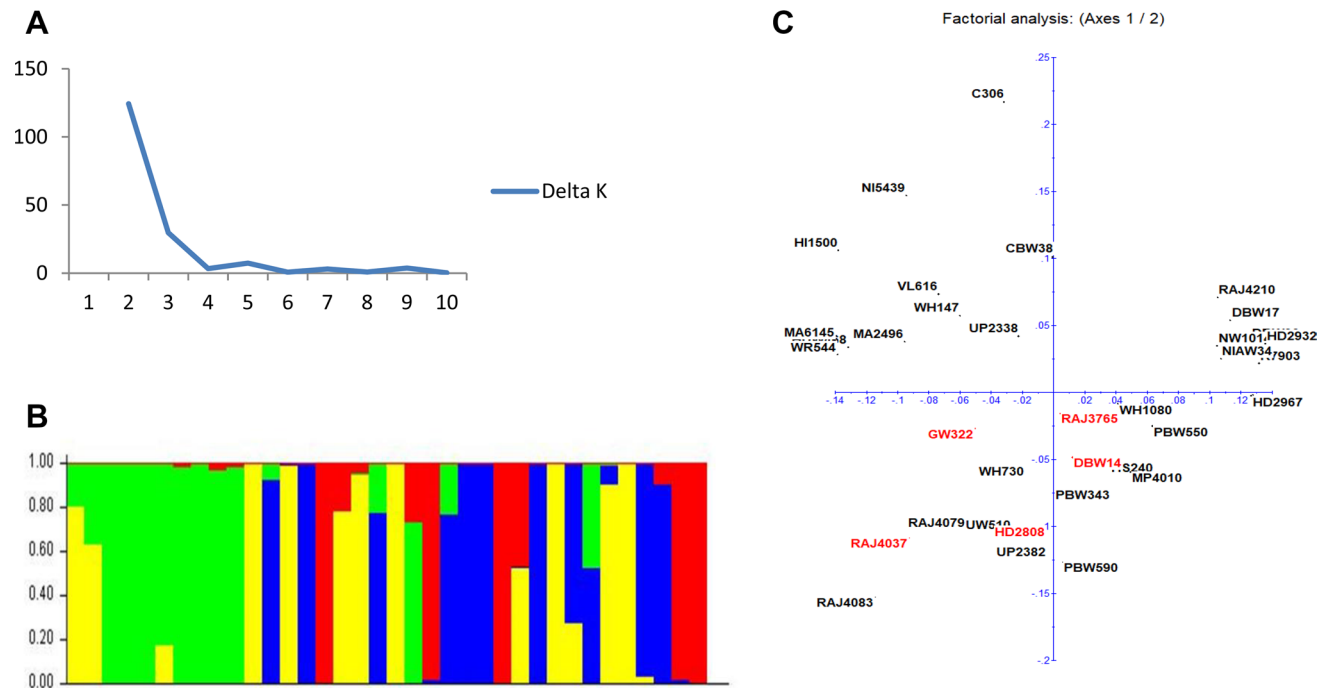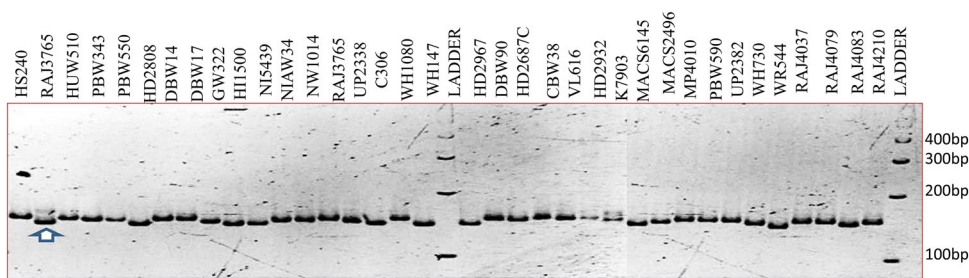

Fig. 4 Estimation of number of groups based on output from "STRUCTURE". **a** △K, **b** Bar plot showing grouping of 36 wheat genotypes into four different groups as shown in different colours, and **c** two – dimensional plot generated from PCA plot. The maximum of adhoc measure △K was found to be K = 4

marker is > 0.5, the marker is considered a highly informative, if the PIC value ranged from 0.25–0.5, the marker is a moderately informative and if the PIC value < 0.25, then the marker is slightly informative marker. Patil et al. [46] observed the average PIC of 0.29 for 47 miRNA-SSRs when screened on pomegranate. While Fu et al. [52] observed the average PIC of 0.43 for 34 miRNA-SSRs when screened six brassica species. The main limitation in genic SSRs is lower level of polymorphism than genomic-SSR markers [53, 54]. In this study, the average of GD and PIC were 0.40 and 0.35, respectively. The PIC value obtained in this study were in close agreement to the mean PIC value of 0.27 estimated previously [55]. The lowest PIC value was observed for PSSR-HSP21 and PSSR-RacB whereas the maximum PIC value was observed for PSSR-GGT. The other cg-SSR markers with high PIC values for PSSR-CHIP targeted TaCHIP (PIC of 0.69), PSSR-HSP97 targeted TaHSP-97 SSR (PIC

of 0.68), PSSR-CML11 targeted TaCML11 (PIC of 0.63) followed by miRNA based SSRs like PSSR-miR160a (PIC of 0.66) and PSSR-miR10 (PIC of 0.65). The PIC values of cg-SSR and miRNA-SSR primers are given in Table 2. Thus, the 37 SSRs used in our study might have potential to serve as functional markers for genetic diversity analysis in wheat. Population structure analysis is useful in understanding mapping and genetic diversity analysis. Wheat genotypes were divided to four groups. The PCoA results were in an agreement with STRUCTURE results. There were five wheat genotypes in group-1, while 11 genotypes in each group-II and group–III followed by nine genotypes in group-IV. HHT genotypes clustered separately in group-I (Fig. 5), followed with mixed pattern for rest of the groups. The data obtained from trait specific SSRs in this study might be helpful for better understanding of the genetic diversity in wheat.

Fig. 5 Dendrogram generated from an unweighted pair group method analysis (UPGMA) cluster analysis based on heat- responsive 37cg-SSR and miR-SSR markers. First cluster showing HHT wheat genotypes in RED colour, while rest of clusters showing mixed MHT and LHT distributions
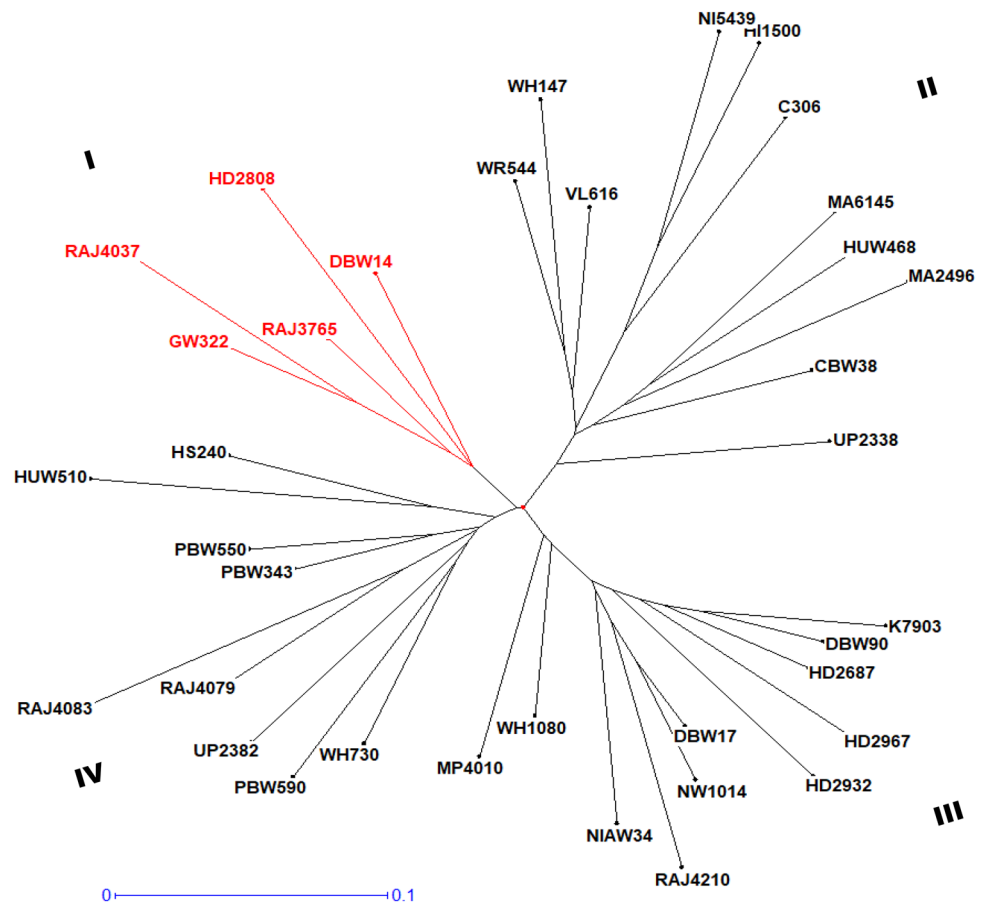
In conclusion, this is first report of development and validation of cost effective and easy to use heat-responsive gene based cg-SSRs and miRNA-SSRs markers to differentiate the wheat genotypes based on their genetic diversity, particularly with response to heat stress. Thus, the identified cg-SSR and miRNA-SSR markers might have a great potential for studying of genetic diversity and relationships in wheat for heat stress tolerance. Upon further validation these markers would be useful in molecular breeding programs, QTL mapping and association studies in wheat.

Author contributions PS conceived the theme of the study and designed the experiment. GM recorded and analysed field data. Shefali did bioinformatics analysis; SKM analysed the genes and SSR data. PS, SKM, SKS and GPS drafted the manuscript. All co-authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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