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RESEARCH ARTICLE

Exploration of Heat Stress-Responsive Markers in Understanding Trait Associations in Wheat

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Abstract

Heat stress (HS) is detrimental to wheat production and productivity globally. To combat HS, several genetic, molecular, and genomic approaches have been employed in the past. Analyzing the physiochemical mechanisms and the important regulatory genes involved is the key to develop HS tolerant plants. In the present work, a total of 243 novel simple sequence repeat (SSR) markers developed from stress-associated genes identified through RNA-seq were used for understanding marker–trait associations. 37 SSRs were found to be clearly polymorphic and among these, 28 SSR loci were significantly associated with component traits of HS tolerance. The polymorphic SSRs were validated for diversity analysis on a subset of 85 genotypes. The genotypes were grouped into four clusters representing diverse and similar alleles imparting HS tolerance in Indian and exotic genotypes. Additionally, 28 genes selected for the expression analysis confirmed that 15 genes were induced under HS in the thermotolerant WH1021 and Raj3765 and repressed in thermosusceptible HD2009 cultivar. Hence, the information on traits associated with candidate genes and the SSR markers overlying on the gene will enhance our understanding of thermotolerance mechanism operating in wheat and will help the breeders in the precise development of heat-tolerant genotypes through marker-assisted selection (MAS).

Keywords Wheat · Ssrs · Association · Gene expression · Diversity · Heat stress

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple food grains on earth. After years of domestication in fertile crescent region, the present day wheat varieties evolved that are adapted to a wide range of environmental conditions ranging from high-humidity regions like South America to low-humidity regions like India, Nigeria, Egypt, and Australia (Pont et al. 2019). Wheat yields are affected by both biotic and abiotic stresses. Among the abiotic stresses, drought and heat are the most severe stresses that affect the life cycle of the crop (Zampieri et al. 2017). These two factors progressively became important due to global climate change (Akbarian et al. 2011; Zampieri et al. 2017). The global temperature has been presumed to increase by 0.18 °C per decade (Hansen et al. 2012). An estimated 6% loss in wheat production occurs globally for every 1 °C rise in temperature (Asseng et al. 2015). Higher temperatures have a direct influence on plant growth and crop yields owing to reduced opportunities for photosynthesis since the life cycle is truncated (Bita and Gerats 2013; Stocker et al. 2013). At grain-filling stages, the rise in temperature adversely affects the quantity and quality of wheat grains thereby inducing various cellular and metabolic changes.

The advances in biotechnology including recent progress in genomics and molecular breeding have enabled wheat researchers to use the technology in mitigating the detrimental effects of HS (Lamaoui et al. 2018). Molecular markers improve the efficiency of conventional plant breeding by indirectly selecting for the gene of interest. Simple

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sequence repeats (SSRs) or microsatellites are efficiently and frequently used for the identification of the quantitative trait loci (QTL) linked to drought and HS tolerance (Pinto et al. 2010; Paliwal et al. 2012; Mondal et al. 2015). Such QTL linked markers hold promise in forward marker-assisted selection for improved selection efficiency and development of stress-tolerant cultivars (Rai et al. 2018; Sandhu et al. 2019). QTLs for various physiological and morpho-agronomic traits have been studied internationally; significant progress has been made in the mapping of QTLs for yield and contributing traits under HS in wheat (Pinto et al. 2010; Sukumaran et al. 2018; Tadesse et al. 2018). However, the use and implementation of QTLs is carried out to a limited extent due to differences in genetic backgrounds, environments and poor understanding of expression and regulation of genes governing the trait. In addition, the small effect of a single QTL enforces the breeder to prioritize for stable and strong effect QTLs which limit the whole efforts (Tricker et al. 2018). A viable option is the development of markers from the genic regions of HS transcriptomes that may help in population genetics and association studies through the identification of genes/QTLs linked to component traits of HS tolerance.

Stress-associated genes are induced in response to heat stress (Chauhan et al. 2011; Rampino et al. 2012; Lamaoui et al. 2018). Though significant progress has been made in research on HS tolerance, information on the genes involved in HS response is limited. In the past decade, most of the studies were restricted to the identification and mapping of QTL/genomic regions for constituent traits of HS tolerance (Pinto et al. 2010; Talukder et al. 2014; Mondal et al. 2015; Tadesse et al. 2018) but stress-associated genes lying in such genomic regions were known to a lesser extent. In the recent past, Acuna-Galindo et al. (2015) identified QTL hotspots in eight major clusters using the meta-QTL approach on linked SSRs and reported a few clusters harboring agronomically important genes. Li et al. (2004) reported that the SSRs lying in the gene regions may be involved in regulating the expression of respective genes. The genic SSR markers from candidate genes have greater potential in identifying marker–trait associations in germplasm collections involving diverse backgrounds and environments. Marker–trait associations of these candidate genes-based SSRs could prove very helpful in future genetic diversity and MAS studies.

Although several gene expression studies have identified differentially expressed stress-responsive genes in contrasting wheat genotypes under HS, yet the association with component traits of HS tolerance still remains to be learned (Qin et al. 2008; Kumar et al. 2017; Li et al. 2019). Keeping this in view, microsatellite markers were developed from the RNA sequencing analysis of Indian heat-susceptible and -tolerant genotypes. A total of 1216 differentially expressed genes (DEGs) were observed that contain more than 2000 SSR motifs. Among these, the stress-associated genes (SAGs) including the chaperones, transcription factors, signaling factors, etc. harbored 243 SSRs. In the present study, we assess the performance of candidate gene-based novel microsatellite markers in understanding marker–trait associations in indigenous and exotic germplasm collections and gene expression pattern of selected genes in contrasting genotypes.

Materials and Methods

Plant Materials

An international core set for abiotic stress comprising of 145 lines, received from CIMMYT, Mexico under the Generation challenge Program was evaluated under timely (TS) and late sown (LS) environments for three years. The experiment was laid out in a randomized complete block design with two replications in three rows of one-meter length each. The core set was used for screening of 243 candidate gene SSRs newly developed from whole transcriptome sequencing of contrasting wheat cultivars under HS.

A subset of 85 lines including 34 lines from this larger international core set and 57 elite Indian lines developed for different agro-ecological conditions were utilized for corroboration of marker–trait associations of identified candidate gene SSR markers. The subset mainly included parents of Indian and International mapping populations for drought and HS tolerance (Table S1). The subset experiment was laid out in alpha-lattice design in two environments (timely and late sown) in the years 2018–19. Each environment has two replications and each replication constitutes 8 blocks. Every block had 11 genotypes. For the TS environment, planting was done during the second week of November with recommended irrigation; while for the LS, planting on a delayed date during first week of January was carried out. The crop was maintained using standard cultivation practices prescribed for wheat.

Development and Scoring of SSR Markers

SSR markers were developed from RNA sequencing data generated on heat stress-tolerant (HD2985) and a heat stress-susceptible (HD2329) genotype (for details see, Kumar et al. 2017). Among the identified SSR motifs, 243 SSRs overlay on the stress-associated genes (SAGs) such as chaperones, transcription factors, signaling factors, etc. Therefore, a total of 243 SSR markers were developed from differentially expressed heat-responsive transcripts obtained under control (22 ± 3 °C) and high-temperature stress (42 °C, 2 h) conditions using the Microsatellite identification tool (MISA; https://pgrc.ipk-gatersleben.de/misa/misa.html). The

sequences, repeat motifs and amplification conditions of 243 SSR markers can be obtained from Kumar et al. (2017). These newly synthesized candidate gene-based SSR markers were evaluated for their performance in 145 lines of the international core set. The PCR reaction profile was: DNA denaturation at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 or 60 °C for 1 min (depending upon primer), 72 °C for 1 min and finally 72 °C for a final extension of 10 min. Amplified PCR products of each reaction were separated on 3% metaphor agarose gel (Lonza, Rockland ME, USA) and were photographed using a Gel Documentation System, by keeping the magnification constant. Manual gel scoring was done for each gel picture based on the bands of the standard 100 bp DNA ladder. Every allele was scored as present (1) or absent (0) for individual SSR marker. The markers that produced the expected size of the amplicon with clear bands and showed polymorphism were further validated on a smaller set of 85 lines.

Phenotypic Characterization of Germplasm Lines

Phenotypic characterization of international core set was carried out in three different growing seasons (2013, 2014, and 2016) during TS and LS conditions for various agronomic and physiological traits. The gross plot size of the TS experiment was 1.38 m × 3.0 m with rows at 20 cm apart, whereas for LS experiments, the gross plot size was 1.08 m × 3.0 m with a row-to-row spacing of 18 cm. Data was recorded for days to heading (DH), days to maturity (DM), plant height (PH), flag leaf area (LA), 1,000 kernel grain weight (TGW), yield per plot (YLD), canopy temperature at vegetative (CT1) and reproductive (CT2) stage, normalized difference vegetation index (NDVI) at vegetative stage (lateboot stage (Z49), NDVI1), grain-filling stage (early milk stage (Z73), NDVI2) and grain maturity stage (late milk stage (Z83–87), NDVI3) according to Zadoks scale (Zadoks, 1974). NDVI at different growth stages was recorded using GreenSeeker® (Trimble, Inc.). Hand-held infrared thermometer (Kane May Model Infratrace 8000, USA) was used for the measurement of CT. Data on DH, DM, CT and NDVI were recorded on a plot basis; whereas PH, LA were recorded on randomly chosen five plants per plot. At maturity, plants were harvested from the experimental plots individually to record the grain yield.

The experiment on a subset population was conducted during 2018–19 in TS and LS environments in alpha-lattice design as described above. Data on various agro-physiological traits viz. DH, grain weight per spike (GWPS), TGW, grain length (GL), grain width (GW), biomass (Bio), yield per plot (YLD), CT, NDVI were recorded in replications in both environments. DH, CT, NDVI, Bio, and YLD were recorded on a plot basis while GWPS and TGW were recorded on randomly collected 20 spikes from each plot. GL and GW were measured for a random sample of grains of each genotype. Grain characteristics were measured using a grain image that was processed using software GrainScan developed by CSIRO (www.plant-image-analysis/software/grainscan).

Phenotypic Data Analysis

Phenotypic data recorded on each genotype of the subset population are subjected to descriptive statistical analysis. The analysis included block, replication, and treatment for each variable. Best Linear unbiased estimates (BLUEs) for the phenotypic data were calculated using R 3.6.0. Analysis of variance on mean values of 2 replications per genotype were analyzed in alpha-lattice design using SAS 9.3. The BLUE values of different traits were further used to perform the correlation analysis.

Genotypic Data Analysis

The model-based (Bayesian) Structure version 2.3.4 was applied to identify clusters of genetically similar individuals on the basis of their genotypic data. The program was run five times independently for *K* value (number of subpopulations) ranging from 1 to 10, adopting the admixture model. The normal logarithm of the probability was calculated against each K value, and the optimal number of subpopulations was determined using the ΔK approach described by Evanno et al. (2005). The threshold for statistical significance was determined by running 10,000 permutations.

To study the candidate gene-based genetic diversity on subset genotypes, the binary data produced from the scoring of bands on randomly chosen nineteen polymorphic SSR markers from above (37 polymorphic markers) were used as input for further analysis. Genotypic data obtained from 85 germplasm lines were used as input in DARwin 6.0 program (https://darwin.cirad.fr/darwin), and a dendrogram was constructed using the unweighted neighborhood joining algorithm.

Marker–Trait Associations

The association of genotypic and phenotypic data was conducted using TASSEL 2.1 software (https://www.maizegenetics.net), via a general linear model (GLM) with 1000 permutations. The significance threshold of the association was determined by the p value (< 0.05). In GLM model, population structure of the germplasm was included as fixed effects, while the association was estimated by simultaneous accounting of the population structure (Q matrix).

qRT-PCR Analysis

Ten-day-old seedlings of three moderate to highly tolerant (Halna, Raj3765, and WH1021) and one susceptible (HD2009) varieties were used for the purpose of qRT-PCR validation of 28 gene specific SSRs. The candidate gene SSRs included: Four SSRs (SSR30, SSR32, SSR35, and SSR36) located in genes encoding heat shock proteins (HSPs), two SSRs (SSR100, SSR155) from gene involved in signaling, nine SSRs (SSR12, SSR13, SSR60, SSR64, SSR79, SSR166, SSR177, SSR179, SSR205) from genes encoding transcription factors (TFs), two SSR (SSR158, SSR223) belonging to gene encoding regulatory proteins and 11 SSR belonging to genes with miscellaneous function.

Total RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) from the control (22 ± 3 °C) and heat shock-treated (42 °C for 4 h) leaf tissues of the four varieties at 10-day-old seedling stage. The first strand cDNA synthesis was performed using Verso cDNA synthesis kit (Thermo Scientific Inc., USA) following the manufacturer's instruction. Expression of the 28 genes was analyzed using real-time PCR with SYBR Premix (ABI, USA). PCR primers were designed using the NCBI Primer design tool (https://www.ncbi.nlm.nih.gov-tools-primer-blast) (Table S1). 18S RNA was used as a reference gene for normalization of expression. The relative expression was determined using the comparative Ct method (Livak and Schmittgen 2001). The relative fold change expressions were calculated for normalized heat shock-treated versus control samples. The change in expression between control and heat stress treatment was statistically analyzed using Student's *t* test at a 5% level of significance.

Results

Screening on International Core Set

A total of 243 genic SSRs were screened on an international core set. Out of 243 SSRs, 93SSRs amplified specific bands; 58 markers produced monomorphic bands and 37 markers were polymorphic. The remaining 148 SSRs did not amplify well and produced nil/fuzzy/multiple bands and were not used for further analysis. The 37 polymorphic SSR markers produced a total of 106 alleles ranging from 2 to 5 alleles per SSR (Table 1). These SSR markers belonged to candidate heat-responsive genes encoding transcription factors (11), heat shock proteins (4), regulatory proteins (7), signaling (3) and others (12) (Table 1).

To understand the association of 37 markers with phenotypic traits, the ancestral contribution of genotypes in the population was estimated; for this purpose data on 145 lines with 37 SSR markers were utilized. The model-based analysis with Structure identified an optimal number of subpopulations at $K = 3$ (Fig S1) as the maximum likelihood when K was set from $K = 1$ to $K = 10$ subpopulations. The number of 145 wheat accessions assigned to each of the three inferred clusters is 58, 36 and 51 when the membership proportion was set at more than 0.5 for each cluster. Fixation index (F_{ST}) values between all groups were significant ($p < 0.001$) suggesting a real difference among these clusters. The inferred cluster values were subsequently utilized for understanding marker-trait associations. The phenotypic data on 8 agronomic traits from different growing seasons (2013, 2014, and 2016) were used for association with SSRs (Table S2). A total of 22 SSR markers were identified to be associated with the 7 traits at the 0.05 probability level (Table 2), and phenotypic variation ranged from 5.11 to 18.01%. SSR markers viz., SSR30, SSR32, SSR35, and SSR36 (gene encoding heat shock proteins) were associated with the physiological traits, NDVI and CT. SSR64 (gene encoding ethylene-responsive TF) was associated with various traits including NDVI, CT and YLD. SSR141 was associated with LA and NDVI in the years 2013 and 2014, and YLD in the year 2016.

Phenotypic and Genomic Analysis on Subset Population

The 37 polymorphic SSR markers identified as above were used for validation on a subset population of 85 lines. The summary statistics of phenotypic data recorded under TS and LS environments on this subset is presented in Table 3. Analysis of the variance indicated significant variations for the majority of the traits (Table 3). The germplasm lines showed difference in adaptive traits under the two environmental conditions. The coefficient of variation (CV) ranged from 4.10 to 18.52 for TS, and 5.71 to 22.4 for LS environments. Heritability ranged from as low as 0.29 for CT to 0.70 for NDVI in LS environments. Significant genotypic differences were observed for all the traits. The average reduction due to HS for agronomically important traits in LS environments can be seen in Fig. 1.

To investigate the level of association of grain yield with various phenotypic traits, the correlation between physiological and agronomic traits with YLD was analyzed under normal and HS conditions (Table 4). There was a positive correlation of YLD with GL, GW, TGW and GWPS under LS environments. NDVI at different intervals showed significant positive correlation with GL, GW, and GWPS in TS and LS condition. A significant negative correlation of DH was observed with GL, GW, GWPS, and TGW under LS. On the other hand, during TS environments CT had a negative correlation with GW and, TGW.

Table 1 List of heat-responsive SSRs, their gene description, repeat motifs and number of alleles amplified in wheat

SSR ID	Gene ID	Gene description	Role	Repeat motifs	No. of alleles
SSR12	IWGSC_CSS_4BL_scaff_6990050:1:3750:1	stress_transcription factor a-1b	TF	(GCA)6	4
SSR13	IWGSC_CSS_2BL_scaff_8050210:1:14,690:1	stress_transcription factor rf2b-like	TF	(CAG)5	5
SSR30	IWGSC_CSS_1AL_scaff_3975644:1:3913:1	heat shock protein 101	HSP	(CGG)5	3
SSR32	IWGSC_CSS_7AS_scaff_4217917:1:8113:1	heat shock protein	HSP	(ACG)6	3
SSR35	TRIAE_CS42_6AS_TGACv1_485621_AA1548950	HSP70	HSP	(TGT)5	4
SSR36	TRIAE_CS42_U_TGACv1_643360_AA2131150	heat shock protein	HSP	(GTC)6	4
SSR50	IWGSC_CSS_3B_scaff_10614606:1:3273:1	expansin expa11	M	(GAA)6	4
SSR54	IWGSC_CSS_7AS_scaff_4230709:1:9177:1	E3 ubiquitin-protein ligase ring1-like	R	(TTCT)5	3
SSR59	TRIAE_CS42_2BL_TGACv1_131330_AA0426150	dehydrin 1	M	(TCC)5	3
SSR60	IWGSC_CSS_2BL_scaff_7993893:1:6513:1	dehydration-responsive element-binding protein	TF	(CCA)6	2
SSR62	IWGSC_CSS_2BS_scaff_5200682:1:27,955:1	plastid omega-3 fatty acid desaturase	M	(CGC)5	2
SSR64	IWGSC_CSS_6AL_scaff_5780850:1:6292:1	ethylene-responsive transcription factor	TF	(GCC)6	2
SSR72	IWGSC_CSS_1AL_scaff_3795905:1:9082:1	early flowering 3	M	(CAA)5	2
SSR73	IWGSC_CSS_7DL_scaff_3319496:1:17,313:1	pollen-specific protein sf21-like	M	(TTC)6	2
SSR76	IWGSC_CSS_4AL_scaff_4AL_7061370:1:12,528:1	barley stem rust resistance protein	M	(CCT)8	5
SSR79	IWGSC_CSS_5BL_scaff_10789552:1:1723:1	wrky transcription factor partial	TF	(CGG)5	2
SSR92	IWGSC_CSS_1BL_scaff_3858292:1:11,020:1	heme oxygenase 1	S	(CGC)5	2
SSR100	TRIAE_CS42_2BL_TGACv1_129772_AA0395400	glutaredoxin	S	(TCC)5	2
SSR122	IWGSC_CSS_6BL_scaff_4398244:1:3993:1	alpha-galactosidase alpha-n-	M	(GA)7	3
SSR131	IWGSC_CSS_7BL_scaff_6739810:1:2252:1	dna-binding protein mnb1b	R	(TGC)5	4
SSR141	TRIAE_CS42_5DL_TGACv1_433651_AA1418390	aba 8-hydroxylase	M	(CGA)5	4
SSR155	IWGSC_CSS_2BL_scaff_8087983:1:24,078:1	-glutaredoxin subgroup i	S	(GAG)5	3
SSR157	IWGSC_CSS_1AS_scaff_3259168:1:13,155:1	ocs element-binding factor 1	R	(CAG)5	2
SSR158	IWGSC_CSS_5BL_scaff_10793275:1:15,180:1	lipid-binding protein	R	(AGTG)5	2
SSR166	IWGSC_CSS_2AL_scaff_6429764:1:12,906:1	fd-like 15 protein	TF	(CTTG)6	2
SSR170	TRIAE_CS42_2BS_TGACv1_146521_AA0467430	alpha-galactosidase alpha-n-	M	(GA)7	2
SSR175	IWGSC_CSS_7BL_scaff_6747596:1:7144:1	nuclease harbi1-like	R	(GGTT)5	2
SSR177	IWGSC_CSS_7DL_scaff_3351966:1:6586:1	golden2-like transcription factor	TF	(CGG)5	5
SSR179	TRIAE_CS42_3B_TGACv1_221666_AA0746950	ap2 protein	TF	(TCC)6	3
SSR183	IWGSC_CSS_5BL_scaff_10805401:1:9312:1	g-type lectin s-receptor-like serine threonine-protein kinase sd2-5-like	R	(GA)6	4
SSR184	TRIAE_CS42_5BL_TGACv1_404628_AA1306740	ring finger protein	TF	(CGG)5	2
SSR205	IWGSC_CSS_2BL_scaff_8008064:1:11,548:1	zinc finger protein	TF	(CGA)5	2
SSR221	IWGSC_CSS_1BL_scaff_3850458:1:18,499:1	NADH dehydrogenase	M	(TGG)5	2
SSR223	IWGSC_CSS_2AL_scaff_6392290:1:6702:1	silencing group b protein	R	(CAC)5	2
SSR230	IWGSC_CSS_5AL_scaff_2781953:1:6260:1	peroxisomal biogenesis factor 11 family protein	M	(TA)6	2
SSR235	IWGSC_CSS_1BS_scaff_3464231:1:11,062:1	gata transcription factor 16-like	TF	(CAG)5(CAA)5	2
SSR240	IWGSC_CSS_4AL_scaff_4AL_7121598:1:5090:1	fructan:fructan 1-fructosyltransferase	M	(GCG)5	4

TF transcription factor, *HSP* heat shock protein, *S* signaling molecule, *R* regulatory protein, *M* miscellaneous

Table 2 Marker–trait associations (R^2) of the 37 candidate gene SSR markers with various agro-physiological traits in international core set during three (2013, 2014 and 2016) different crop seasons under late sown conditions

Trait	2013			2014			2016		
	Locus	*p* value	R^2	Locus	*p* value	R^2	Locus	*p* value	R^2
LA	SSR141	0.049	0.0821	SSR141	0.0482	0.0824	–	–	–
NDVI 1	SSR59	0.0437	0.083	SSR59	0.0203	0.0548	SSR12	3.28E–118	0.9448
	SSR60	0.0224	0.0606	SSR141	0.0092	0.1109	–	–	–
NDVI 2	SSR64	0.0108	0.0582	SSR223	0.0056	0.1049	SSR13	0.0292	0.0538
	SSR141	0.0184	0.0995	SSR141	0.0276	0.0909	SSR155	1.52E–05	0.1801
NDVI 3	SSR64	0.0176	0.0507	SSR30	0.0322	0.0781	–	–	–
DH	SSR60	0.0224	0.0616	SSR60	0.0224	0.0616	SSR50	0.0025	0.1052
	SSR79	0.0052	0.0739	SSR79	0.0052	0.0739	SSR155	1.23E–05	0.1789
	SSR166	0.0309	0.0623	SSR166	0.0309	0.0623	–	–	–
DM	SSR50	0.0408	0.0603	SSR50	0.0408	0.0603	–	–	–
CT1	SSR50	0.0138	0.0807	SSR50	0.0138	0.0807	SSR13	2.97E–04	0.1143
	SSR35	0.0079	0.094	SSR35	0.0079	0.094	SSR32	1.42E–08	0.2371
	SSR122	0.0135	0.0725	SSR122	0.0135	0.0725	SSR76	9.32E–09	0.1276
	SSR221	0.0229	0.0531	SSR221	0.0229	0.0531	SSR155	1.68E–05	0.1779
CT2	SSR59	0.0341	0.0861	SSR59	0.0466	0.0792	SSR155	0.0046	0.0988
	SSR60	0.0284	0.0547	SSR60	0.0058	0.0767	SSR240	0.0301	0.0677
	SSR64	7.17E–05	0.1261	SSR64	1.42E–04	0.1148	–	–	–
	SSR122	3.85E–05	0.1397	SSR122	1.68E–04	0.1198	–	–	–
	SSR179	0.0251	0.054	SSR35	0.0316	0.0678	–	–	–
	SSR221	1.10E–04	0.1149	SSR221	7.42E–04	0.0891	–	–	–
YLD	SSR64	0.0104	0.0583	SSR64	0.0144	0.0518	SSR12	0.0061	0.0704
	SSR122	0.0217	0.0666	–	–	–	SSR36	0.0294	0.1351
	–	–	–	–	–	–	SSR76	0.0029	0.1689
	–	–	–	–	–	–	SSR141	0.0194	0.0995
	–	–	–	–	–	–	SSR177	0.0113	0.0743
TGW	SSR122	0.0088	0.079	SSR122	0.0087	0.0793	SSR36	0.0063	0.1637
	–	–	–	–	–	–	SSR205	0.009	0.0503

LA flag leaf area, *NDVII* normalized difference vegetation index at lateboot stage (Z49), *NDVI2* at grain-filling stage Z73, *NDVI3* at grain maturity stage (Z83–87), *DH* days to heading, *DM* days to maturity, *CT1* canopy temperature at vegetative stage, *CT2* at reproductive stage, *YLD* yield per plot, *TGW* 1000 kernel grain weight

Diversity analysis Using Candidate Gene SSRs

The polymorphic SSR markers obtained as above in the core set were used for generating polymorphism profiles of the selected subset population. A total of 50 alleles were generated with an average of 2.6 alleles per locus. The allele diversity data was used to estimate dissimilarity which was subsequently used for cluster analysis. Four major clusters were produced; Cluster I and II were predominantly represented by genotypes of Indian origin with the exceptions of Frontana, Chiriya3, Giza163, Baviacora, synthetic lines, and UASD lines whereas Cluster III involved the majority of the exotic genotypes representing HS tolerant cultivars (Fig. 2). Cluster IV comprised of only six genotypes; two among these were exotic.

Marker–Trait Associations

The allele diversity data of the subset population were utilized in estimating population structure for inferring marker–trait associations subsequently. The k was assumed from 1 to 10 subpopulations using the co-ancestry model. The estimated linkage probability revealed that eighty-five germplasm lines were grouped into two clear subgroups (Fig. 3). The genotypes with more than 70% of the proportion of similar genome (based on shared alleles) were assigned to a common subgroup. One group (Red) represented mostly exotic lines from Australia and CIMMYT and the other group (Green) represented lines from Indian wheat breeding programs (Fig. 3). The lines G5, G12, G16, G31, G32, G34, G46, G6, G62, G65, G70, G74,

Table 3 Analysis of variance and basic statistics for phenotypic traits under timely (TS) and late sown (LS) conditions in wheat

	TS (Timely sown)						LS (Late sown)					
	Heritability	Genotype variance	Residual variance	Grand mean	LSD	CV	Heritability	Genotype variance	Residual variance	Grand mean	LSD	CV
DH	0.80	33.95	17.1820	99.18	5.23	4.18	0.55	4.8040	28.1589	85.344	3.760	6.218
NDVI1	0.46	0.00041	0.0010	0.75	0.03	4.10	0.48	0.0014	0.0029	0.667	0.053	8.144
NDVI2	0.44	0.00048	0.0012	0.73	0.03	4.80	0.71	0.0031	0.0025	0.601	0.060	8.357
NDVI3	0.52	0.00116	0.0021	0.67	0.05	6.89	0.70	0.0066	0.0057	0.456	0.090	16.536
CT1	0.49	1.27503	2.6694	19.69	1.62	8.30	0.29	0.3592	1.7422	23.091	1.004	5.716
CT2	0.43	0.73505	1.9514	19.51	1.29	7.16	0.35	0.7681	2.8499	27.806	1.409	6.071
GWPS	0.49	0.07827	0.1629	2.19	0.41	18.43	0.58	0.0757	0.1094	1.559	0.363	21.224
Bio	0.47	29,199.29	6585.93	1520.68	248.88	16.88	0.60	28,566.6886	38,591.3781	1111.139	217.058	17.680
YLD	0.72	7991.17	6251.51	426.89	96.20	18.52	0.66	4145.8465	4341.1556	293.121	74.686	22.478

DH days to heading, *NDVI1* normalized difference vegetation index at lateboot stage (Z49), *NDVI2* at grain-filling stage Z73, *NDVI3* at grain maturity stage (Z83–87), *CT1* canopy temperature at vegetative stage, *CT2* at reproductive stage, *GWPS* Grain weight per spike, *Bio* biomass, *YLD* yield per plot, *LSD* least significant difference, *CV* coefficient of variation

and G77 showed less than 70% majority with any single group and, therefore, carried mixed proportion of alleles both from Indian and exotic origin.

A total of 30 marker–trait associations was identified with 14 different SSR markers in both the environments using days to heading as a covariate (Table 5). The average phenotypic variation (R^2) value ranged from 5.2 to 25.1% in the population. Few candidate gene SSR markers were associated with more than a single trait; SSR13 (stress transcription factor) was closely associated with CT, GL, GW, GWPS; SSR76 (barley stem rust resistance protein) was associated with NDVI1, NDVI3 and GWPS; SSR230 (peroxisomal biogenesis factor 11) was associated with GL under TS, LS and YLD under LS. Candidate genes belonging to SSR72, SSR73, SSR100, SSR158, SSR166, SSR170 were also associated with more than one trait. Five markers viz. SSR12, SSR50, SSR60, SSR205, and SSR240 associated with one trait; Bio, CT, Bio, Yld and CT, respectively, in only one (either TS or LS) environment (Table 5).

Expression of Genes Encoding Heat-Responsive SSRs

Among the SSR overlaying candidate genes, 28 candidate genes that associated significantly with important phenotypic traits (Tables 2 and 5) were selected for analyzing expression. Two genes encoding SSR141 and SSR205 did not produce single amplification products in qRT-PCR and were not included in further analysis. The details of qRT-PCR expression analysis are as follows: (i) The two signaling genes harboring SSR100 and SSR155 were significantly upregulated in thermotolerant lines compared to the thermosensitive cultivar HD2009 (Fig. 4) (ii) Heat shock protein (HSP) genes harboring SSR30, SSR32, SSR35, SSR36, had higher expression in the tolerant lines, WH1021 and Raj3765 (iii) TFs carrying SSR12, SSR60, SSR64, SSR166, and SSR179 and genes overlaying SSR73, SSR122, SSR170 and SSR240 had higher expression in the thermotolerant lines (iv) Genes overlaying SSR13, SSR50, SSR76, SSR79, SSR158, SSR177, and SSR221 had low expression under HS in all the four lines (v) the remaining four genes (SSR59, SSR72, SSR223, SSR230) were neither over-induced in tolerant cultivars nor highly reduced in susceptible cultivar under HS. Overall, the genes displayed lower expression in the susceptible line, HD2009, and medium tolerant line, Halna, with an exception of genes carrying SSR12, SSR32, SSR36, SSR64, and SSR179. In Halna, these five genes displayed higher expression under HS. Despite our repeat efforts, no amplification was observed for Raj3765 and WH1021 for the candidate genes carrying SSR179 and SSR76, respectively.

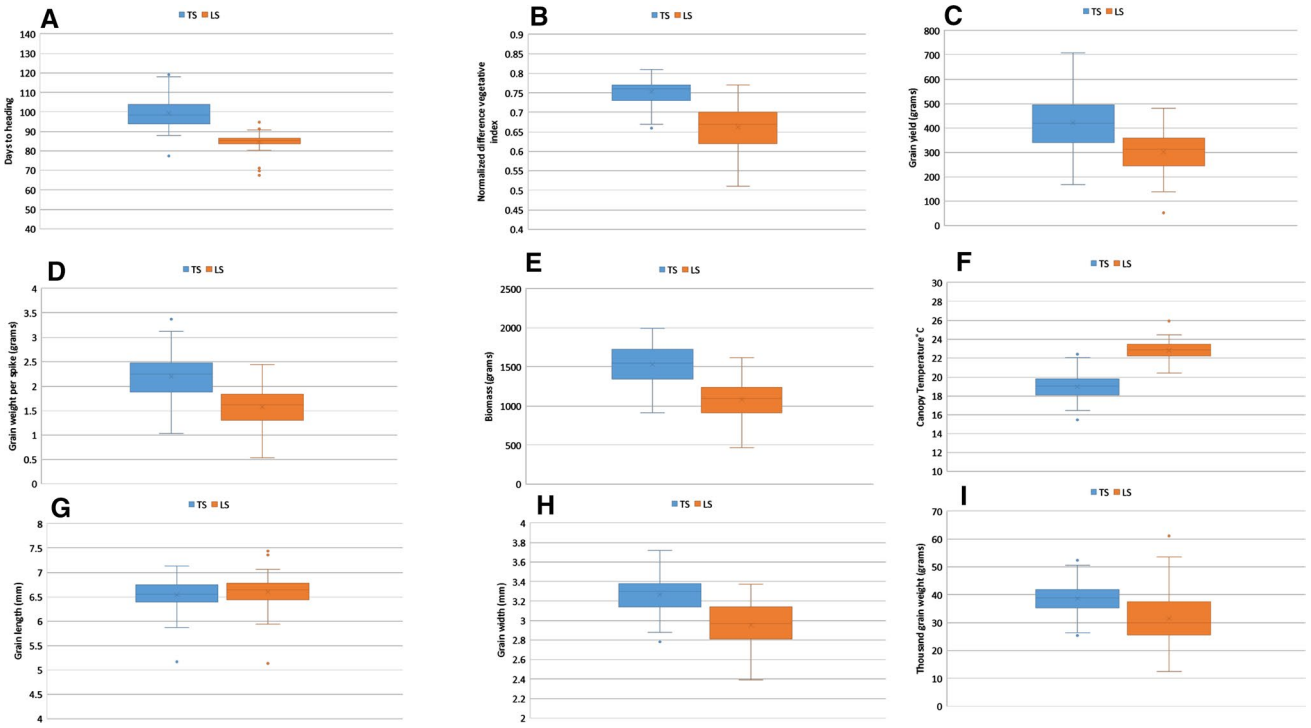

Fig. 1 Box-plots representing best linear unbiased estimates (BLUEs) for mean data on various traits in timely sown (control) and late sown (heat stress) environments. **a** Days to Heading. **b** Normalized difference vegetation index. **c** Grain yield. **d** Grain weight per spike. **e** Biomass. **f** Canopy temperature. **g** Grain length. **h** Grain width. **i** Thousand grain weight. In the timely sown environment, sowing was carried out in mid-November and in the late sown environment, sowing was carried out in first week of January

Table 4 Correlation coefficients among various phenotypic traits under timely (TS) and late sown (LS) conditions. The significance of correlation is depicted by *($p > 0.05$) and **($p > 0.01$)

LS/TS	DH	NDVI1	NDVI2	NDVI3	CT1	CT2	GWPS	Bio	YLD	GL	GW	TGW
DH	1	0.404**	0.579**	0.543**	− 0.157	− 0.174	− 0.100	0.028	− 0.119	− 0.182	− 0.125	− 0.059
NDVI1	0.199	1	0.440**	0.482**	− 0.072	− 0.043	− 0.141	0.167	0.031	− 0.152	− 0.221*	− 0.150
NDVI2	0.020	0.573**	1	0.641**	− 0.111	− 0.114	0.026	0.196	− 0.008	− 0.093	− 0.065	0.044
NDVI3	0.038	0.469**	0.576**	1	− 0.109	− 0.208	0.141	0.132	− 0.026	− 0.078	0.141	0.151
CT1	− 0.097	− 0.052	0.183	0.179	1	0.158	− 0.050	− 0.109	0.003	0.180	− 0.171	− 0.022
CT2	− 0.045	0.125	− 0.012	− 0.061	− 0.260*	1	− 0.055	− 0.114	− 0.009	0.087	− 0.261*	− 0.225*
GWPS	− 0.254*	0.151	0.227*	0.433**	0.108	− 0.002	1	0.276**	0.25*	0.188	0.541**	0.508**
Bio	− 0.117	0.067	0.060	0.183	0.042	− 0.101	0.204	1	0.459**	0.026	0.161	0.222*
YLD	− 0.017	0.107	0.158	0.097	0.003	− 0.198	0.302**	0.081	1	0.078	− 0.086	− 0.015
GL	− 0.288**	0.084	0.224*	0.064	0.289**	− 0.058	0.244*	− 0.024	0.395**	1	0.252*	0.557**
GW	− 0.404**	− 0.001	0.276**	0.402**	0.253*	− 0.064	0.614**	0.185	0.358**	0.362**	1	0.82**
TGW	− 0.215*	0.060	0.129	0.082	0.166	0.021	0.333**	0.316**	0.305**	0.260*	0.339**	1

DH days to heading, *NDVII* normalized difference vegetation index at lateboot stage (Z49), *NDVI2* at grain-filling stage Z73, *NDVI3* at grain maturity stage (Z83–87), *CT1* canopy temperature at vegetative stage, *CT2* at reproductive stage, *GWPS* Grain weight per spike, *Bio* biomass, *YLD* yield per plot, *GL* grain length, *GW* grain width, *TGW* 1000 kernel grain weight

Discussion

Heat stress has a huge consequence in limiting the total grain yield of wheat (Hatfield and Prueger 2015). High temperature during grain-filling stages induces a number of cellular and metabolic changes leading to reduced production of normal proteins. Plants employ various stress adaptive mechanisms to cope up with elevated temperatures. It has been widely accepted that morpho-physiological and

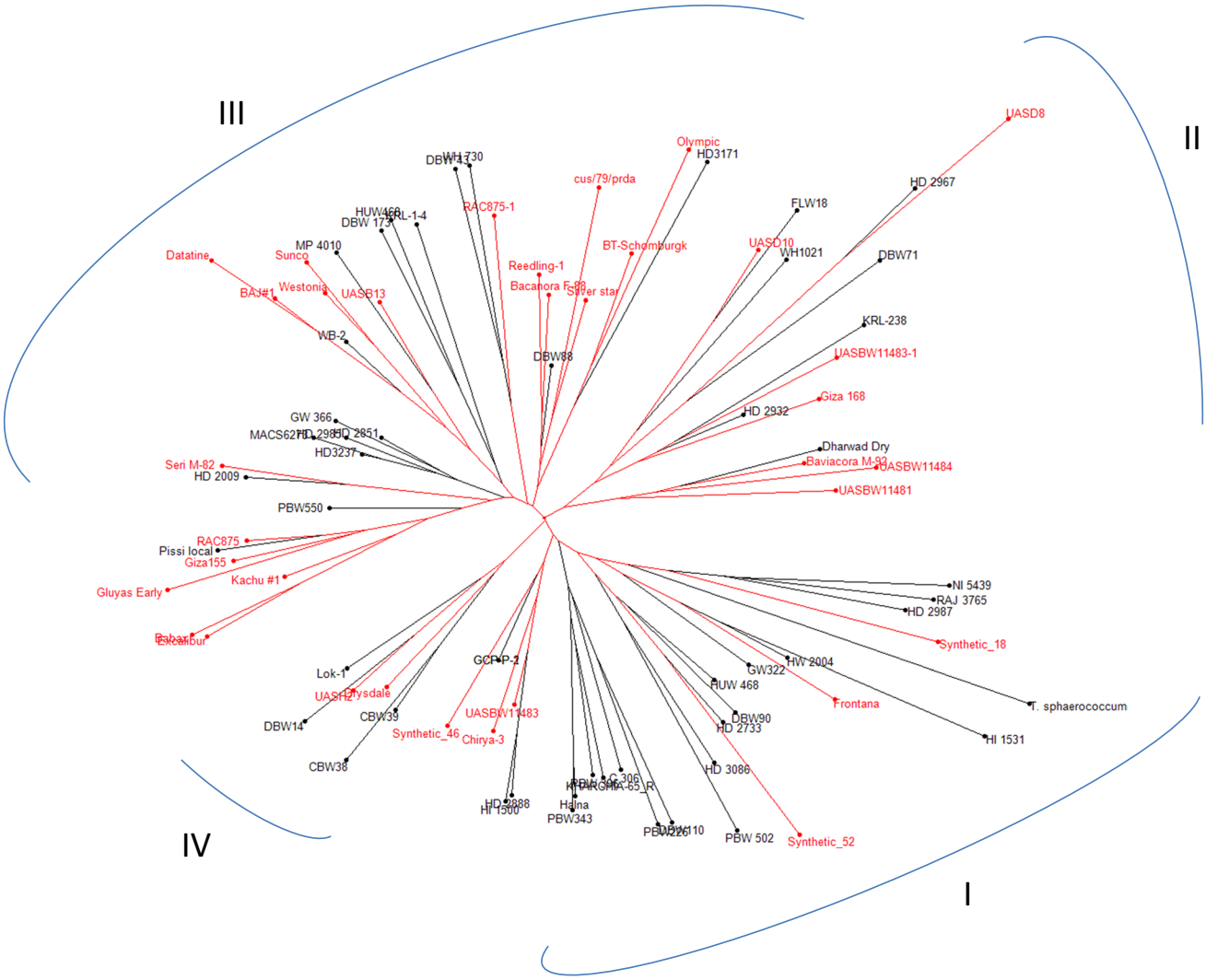

Fig. 2 Neighbor joining tree representing genetic relationships of 85 genotypes using candidate gene-based SSR markers. Four clusters were produced that represent exotic genotypes in red color and indigenous genotypes in black color

yield contributing traits such as DH, DM, NDVI, chlorophyll content, chlorophyll fluorescence, CT, spikelets/spike, grain number/spike, spike length, biomass, tillers/plant and, harvest index had high correlation and heritability under HS and, thus can be effectively used in the breeding program as selection criteria for improvement of stress tolerance and for selection of best genotypes (Araus et al. 2008; Reynolds et al. 2001; Gupta et al. 2017; Pinto et al. 2010; Jain et al. 2018). We observed a high correlation between the majority of the studied morpho-physiological and component traits of yield under HS. Plants tend to have early anthesis and early maturity to avoid the effect of HS (Mondal et al. 2016). In the present study, genotypes showed early heading and a negative association of DH to yield and contributing traits under LS. Grain morphology is also found to be an important parameter under stress conditions. GW had a positive correlation with GL and a negative correlation with DH (Table 3). Kushwaha et al (2011) suggested that heat stress during the terminal stage of the crop growth inhibits the starch biosynthesis which leads to reduction in normal grain size.

Availability of superior and diverse alleles/genes is the starting point of genetic enhancement of crop plants including wheat, for the development of improved cultivars (Abouzied et al. 2013). In the present study, a large proportion of the newly developed candidate gene SSRs had monomorphic alleles (58), implicating that the genes are either conserved or involved in housekeeping among both the susceptible and tolerant genotypes. For the remaining polymorphic SSRs, the subpopulation structure grouped individuals based on their shared alleles into two subpopulations, differentiating genotypes of exotic origin from the Indian origin. This suggests the use of diverse germplasm in bringing out tolerant genotypes at International breeding programs and

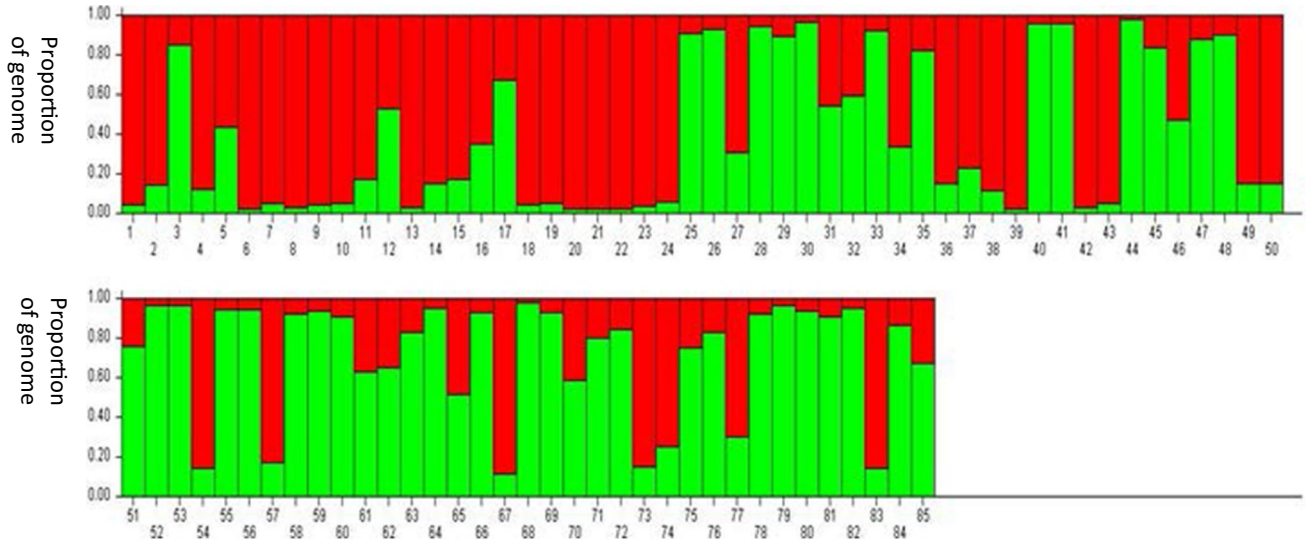

Fig. 3 Population structure of 85 germplasm lines using allele diversity data on polymorphic SSRs. The proportion of genome assigned to each subpopulation is depicted on Y-axis. The bars in red and green colour for each genotype depict the proportion of genome assigned to the two subpopulations. Subpopulation1 (red) comprised of majority of exotic genotypes while subpopulation II (green) comprised of majority of Indian genotypes. The numbers 1–85 represents the serial number of the genotype given in Online Resource1 in the same order

Table 5 Marker–trait associations (R^2) of the identified polymorphic SSR markers with the agro-physiological traits in subset population of 85 genotypes

Trait	LS (Late sown)			TS (Timely sown)		
	Locus	*p* value	R^2	Locus	*p* value	R^2
CT1	SSR13	0.0174	0.0731	SSR73	0.0138	0.0856
	–	–	–	SSR50	0.0028	0.1269
	–	–	–	SSR13	0.046	0.0525
CT2	SSR170	0.0192	0.0897	SSR240	0.0429	0.1018
	SSR13	0.0115	0.0825	–	–	–
NDVI1	SSR158	0.0447	0.0542	SSR166	0.0124	0.1909
	SSR76	0.0149	0.1758	–	–	–
NDVI3	SSR100	0.0287	0.0633	–	–	–
	SSR76	0.0083	0.1973	–	–	–
GL	SSR13	0.0011	0.127	SSR73	0.0307	0.0632
	SSR230	3.57E–06	0.2326	SSR166	0.0225	0.1886
	–	–	–	SSR230	1.12E–06	0.2515
GW	SSR13	0.009	0.0847	–	–	–
GWPS	SSR13	0.0361	0.0587	SSR13	0.018	0.0753
	–	–	–	SSR76	0.0246	0.1688
Bio	SSR72	0.0137	0.0888	–	–	–
	SSR60	0.0045	0.1026	–	–	–
	SSR12	0.0301	0.0621	–	–	–
	SSR170	0.0482	0.0658	–	–	–
YLD	SSR158	0.0349	0.0621	SSR72	0.0421	0.0632
	SSR230	0.0216	0.0674	SSR205	0.000351	0.2214
	–	–	–	SSR100	0.0095	0.0907

NDVII normalized difference vegetation index at lateboot stage (Z49), *NDVI3* at grain maturity stage (Z83–87), *CT1* canopy temperature at vegetative stage, *CT2* at reproductive stage, *GWPS* Grain weight per spike, *Bio* biomass, *YLD* yield per plot, *GL* grain length, *GW* grain width

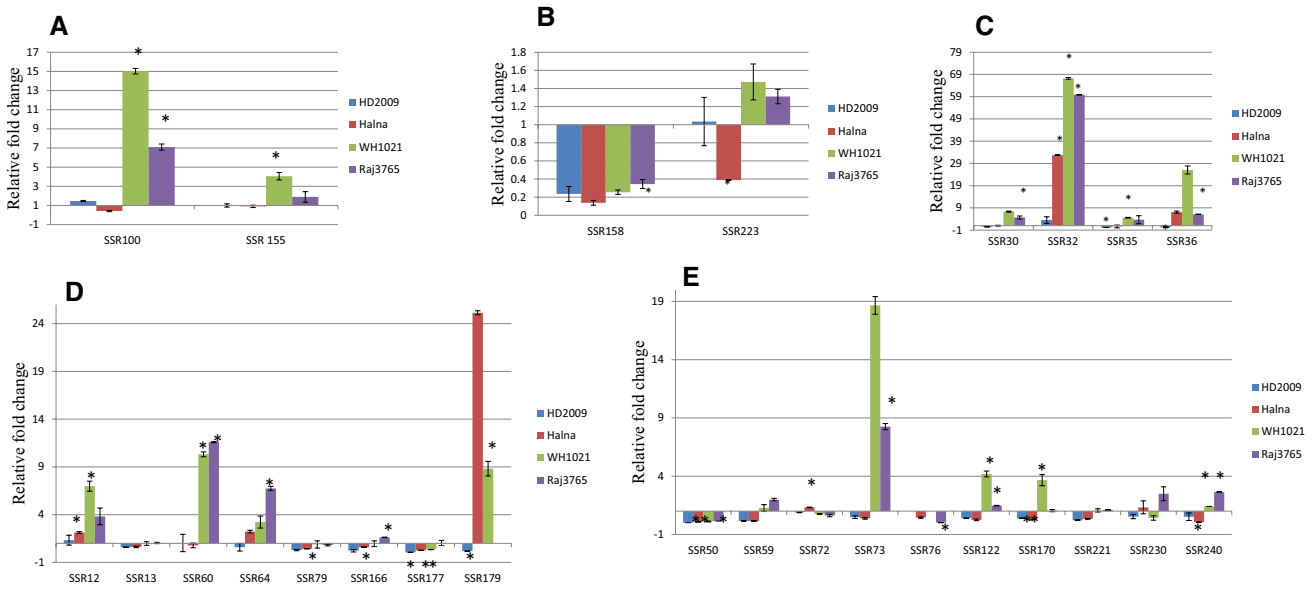

Fig. 4 Relative expression of the SSR harboring genes belonging to signaling (**a**), Regulatory proteins (**b**), HSPs (**c**), TFs (**d**), miscellaneous functions (**e**) in four wheat genotypes: HD2009 (thermosusceptible), Halna (medium tolerant), WH1021 (thermotolerant) and, Raj3765 (thermotolerant). For normalizing the data, the expression of the 18SRNA gene was used; 10-day-old untreated control (C) samples at 22 °C and treated samples (T) with heat shock at 42 °C for four hours were used for estimation of fold change in each genotype; vertical bars indicate SE. Comparison of means between control and treatment samples was carried out by Student's *t* test and significant differences ($p < 0.05$) are represented by asterisk for each genotype

that of the Indian breeding programs. Lines from Australia and CIMMYT formed a distinct subpopulation representing diverse alleles imparting tolerance; however, exotic lines that have been used so far in Indian crossing program shared common alleles and were grouped with the Indian subpopulation. For instance, Kauz is a heat-tolerant genotype from CIMMYT and the lines carrying Kauz in their pedigree such as DBW173, DBW88, MACS6273, Baj and Kachu grouped together in cluster III suggesting the presence of common alleles. The neighbor joining cluster-based findings were in total agreement with the population structure analysis which suggested two subpopulations (exotic and Indian) based on > 70 percent of shared ancestry among individuals. Clusters I and II majorly comprised of heat-tolerant Indian genotypes including HD2932, WH1021, Raj3765, Halna, DBW71, DBW90 and PBW226. These Indian genotypes can themselves serve as a source as parents and could be used in breeding programs without much effort for adaptation. In cluster III, Indian and exotic genotypes did not form separate clusters, which reflect upon the similar expression of stress-responsive genes under HS.

Abiotic stress is a complex process that involves several factors like secondary metabolites, hormones, transcription factors and signaling systems (Lamaoui et al. 2018). Conventional breeding has had limited success in improvement for heat tolerance. Breeding for heat stress is a tough process as the component traits are quantitative in nature, hence the use of MAS and QTL mapping approaches may prove helpful (Collins et al. 2008). Information on QTL hotspots with significant marker-trait associations is being generated in several crops including wheat (Sukumaran et al. 2018; Tadesse et al. 2018; Sinha et al. 2018; Acuna-Galindo et al. 2015). The probability of finding significant marker–trait associations is further enhanced by the availability of genic SSRs. The markers identified from heat stress transcriptome revealed high phenotypic variance in this study either for the same or other correlated traits under stress. As many as 28 SSR loci revealed significant associations with various phenotypic traits. The marker-trait associations were further supplemented with information on heat-responsive genes that are induced under stress. A synergistic response of genes belonging to TFs, HSPs, signaling molecules is observed suggesting specific genomic locations for adaptation and acclimation.

Although transcriptomes have majorly been used for expression analysis, a number of SSRs derived from transcriptome sequencing had been extensively used in plant genetic diversity analyses such as in pigeon pea (Dutta et al. 2011) and chickpea (Kant et al. 2017). SSRs derived from transcriptional approaches are highly suitable for assessing functional diversity. In this study, the polymorphic genic SSRs from diverse TFs, HSPs, signaling and regulatory molecules were associated with important phenotypic traits. Such functional SSRs may regulate gene expression and function under HS. The expression of as many as 15 genes (SSR12, SSR30, SSR32, SSR35, SSR36,

SSR60, SSR64, SSR73, SSR100, SSR122, SSR155, SSR166, SSR170, SSR179, and SSR240) were significantly induced under HS in either of the two thermotolerant lines compared to control (Fig. 4). Expression of only five genes (SSR12, SSR32, SSR36, SSR64, and SSR179) was elevated in Halna under high temperature. In fact, Halna is a medium tolerant line that follows the mechanism of heat avoidance by early completion of its life cycle and hence poor expression of candidate genes. The expression of the majority of the genes was reduced in the HD2009 suggesting under-expression of TFs, HSPs and regulatory molecules that might have led to lower expression of associated traits upon high-temperature stress. Heat stress-responsive TFs and proteins are known to be induced in wheat in several previous reports (Zhang et al. 2017; Xue et al. 2015; Kumar et al. 2017). Heat shock factor C2a was involved in heat protection in developing grains in wheat (Hu et al. 2018). Not only an individual gene is expressed but also a cluster of genes is expressed in response to stress that signifies the presence of several loci in a bonafide QTL. In the tolerant lines WH1021 and Raj3765, genes for HSPs, DREB transcription factor, ethylene-responsive TF, ring finger proteins and sugar metabolism genes were elevated coherently for adaptation to stress. Either such genes or the associated traits may be selected for precise improvement under heat stress.

Candidate genes harboring the newly developed SSR markers were linked to various phenotypic traits in this study. This knowledge of candidate genes governing phenotypic traits will help in a better understanding of the underlying mechanism for HS tolerance which is important to address the issue of heat stress through the use of molecular technology and MAS. The information generated on the traits governed by stress-associated genes is useful for the plant breeders who might be indirectly selecting for the superior traits/genes. The candidate gene-based SSRs have potential use in the transfer of traits for improvement of future breeding programs on HS tolerance.

Author Contributions Conception and Design of experiments was done by NJ, HK, PKS, GPS. Material preparation and data collection was carried out by MPB, NS, DC, PK, RRK. HK, RRK and MPB performed data analysis. Manuscript draft was prepared by MPB and NJ.

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Compliance with Ethical Standards

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

Ethical Approval The article does not contain any studies with human participants or animals performed by any of the authors.

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