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Molecular investigations on grain filling rate under terminal heat stress in bread wheat (*Triticum aestivum* L.)

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Grain yield under post anthesis high temperature stress is largely influenced by grain filling rate (GFR). To investigate molecular basis of this trait, a set of 111 recombinant inbred lines (RILs) derived from Raj 4014, a heat sensitive genotype and WH 730, heat tolerant cultivar was phenotyped during 2009-2010 and 2010-2011 crop seasons, under field conditions. The difference in GFR (dGFR) between the timely and late sown conditions was used as a phenotypic parameter to find association with molecular markers, as parental lines exhibited significant difference for this trait. The mapping population showed clear-cut segregation pattern for differences in GFR between timely and late sown conditions. About 75% of the progenies showed no difference while 25% showed significant difference in GFR under high temperature stress created by late sown condition. To study the association of this trait with the markers, the parental lines were screened with 300 simple sequence repeat (SSR) microsatellite markers out of which 15% (45) were polymorphic between parental lines. These polymorphic markers were utilized for genotyping a subset, comprising of 43 RILs that had clear contrasting variation for dGFR. Regression analysis revealed significant association of dGFR of RILs with two markers viz., Xbarc04 and Xgwm314 with coefficients of determination (R^2) values of 0.10 and 0.06, respectively.

Key words: Grain filling rate (GFR), simple sequence repeat (SSR), heat tolerance, wheat.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops of the world. Due to its wider adaptability, it can be grown under diverse agro-ecological conditions ranging from temperate to subtropical climates. Thus, considerable climatic differences in temperature and relative humidity exists in these areas and wheat crop experiences wide seasonal variations which causes large annual fluctuations in the yield (Munjal and Dhanda, 2005). The exposure of wheat crop to sub-optimal temperatures at establishment and supra-optimal tempe-

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Abbreviations: MAS, Marker assisted selection; **QTLs,** quantitative trait loci; **GWE,** grain weight per ear; **GNE,** grain numbers per ear; **TGW,** thousand grain weight; **GFR,** grain filling rate; **DH,** days to heading; **DA,** days to anthesis; **DM,** days to maturity; **GFD,** grain filling duration; **SGW,** single grain weight; **CTAB,** N-cetyl-N, N, N-trimethyl ammonium bromide; **EDTA,** ethlenediaminetetracetic acid; **RILs,** recombinant inbred lines; **dGFR,** difference in grain filling rate.

Table 1. Pedigree details of the parental lines.

Genotype	Source of genotype	Pedigree
Raj 4014	Durgapura, Jaipur, India	DL 8025/K 9011
WH 730	HAU, Hisar, India	CPAN 2092 / Improved Lok-1

ratures at reproductive phases results in reduced number of grains per spike and poor grain filling leading to shriveled grains with low test weight (Shpiler and Blum, 1991; Nagrajan and Rane, 2002). The proximity to the equator and the popular cropping systems, which involve late sowing of wheat in India, expose wheat to high temperatures (exceeding 35°C) during grain filling (Rane et al., 2000).

Wheat productions under late sown conditions in India and Mediterranean environments (both experience high temperature during grain filling) is substantially low, due to heat stress during grain filling (Tewolde et al., 2006). High temperatures shorten the grain filling period significantly in all the bread and durum wheat genotypes, because of significant interaction of each genotype with temperature (Dias and Lidon, 2009).

Heat-tolerance is a polygenic trait and different components of tolerance, controlled by different sets of genes, are critical for heat tolerance at different stages of development or in different tissues (Howarth, 2005). Thus, the use of correlation and co-segregation analysis, and molecular marker techniques in genetic stocks with different degrees of heat tolerance are promising approaches to dissect the genetic basis of thermotolerance (Maestri et al., 2002). Because of the general complexity of abiotic stress tolerance and the difficulty in phenotypic selection for tolerance, marker assisted selection (MAS) has been considered as an effective approach to improve plant stress tolerance. The use of this approach, however, requires identification of genetic markers that are associated with genes or quantitative trait loci (QTLs) affecting whole plant stress tolerance or individual contributing components. QTL analysis based on high density molecular linkage maps has become a powerful tool for dissecting the genetic basis underlying complex traits into individual components. Up to now, QTL for grain yield and yield components such as grain weight per ear (GWE), grain numbers per ear (GNE) and thousand grain weight (TGW) among others, have been previously reported in wheat by several studies (Huang et al., 2006; Kumar et al., 2006). Quantitative and molecular characterization of heat tolerance in hexaploid wheat has also been reported (Yang et al., 2002). In wheat, QTL analyses have been conducted to map agronomically important characters (Campbell et al., 2003), yield components (Kato et al., 2000). The grain filling rate (GFR) plays a significant role in the final yield of wheat (Beiquan and Kronstad, 1994) and is positively associated with final grain weight. High temperature also increases the rate of grain filling to compensate for shortened grain growth period. However, increase in GFR fails to compensate for the shortened duration and leads to an overall reduction in grain size (Asseng et al., 2011; Wardlaw and Moncur, 1995). Therefore, the selection of genotypes with high GFR appears to be a successful strategy for increasing grain yield under stress conditions. The physiological mechanisms by which crops regulate GFR have been widely explored (Egli et al., 1989), but little is known until now about the genetics and loci affecting this complex trait. Availability of molecular maps enables to dissect complex quantitative traits into component loci and study their relative effects on a specific trait in a segregating population by QTL analysis (Roder et al., 1998). However, up to now, QTL for heat tolerance at GFR in wheat have not been reported, whereas QTL for GFR located on chromosome 4A in wheat under drought stress have been reported (Kirigwi et al., 2007; Wang et al., 2009).

MATERIALS AND METHODS

Plant material

The mapping population Raj 4014 / WH 730 (Table 1) was developed by a single seed descent (F8) from a cross of Raj 4014 (high temperature sensitive) (Annual Report, 2010-11, DWR) and WH 730 (high temperature tolerant) (Kundu et al., 2010) and used to study the effects of high temperature stress on the grain growth. The recombinant inbred lines (RILs) of the Raj 4014 / WH 730 mapping population were assessed for difference in grain filling rate (dGFR) during crop seasons, 2009-2010 and 2010-2011.

Field trials and trait evaluation

The experiments were conducted in the sandy loam soil at research fields of the Directorate of Wheat Research, Karnal (29°C43' N, 76°C48' E, 245 m) India during 2009 to 2010 crop season. Under the study, 111 RILs along with the parents were phenotyped under timely (normal) and late (terminal heat stress) conditions. Data on phenological traits days to heading (DH), days to anthesis (DA), days to maturity (DM) and grain filling duration (GFD)] and grain traits such as grain weight/spike, grain number/spike, TGW among others were recorded. The phenological traits were recorded at 50% stage. GFD was calculated as period from anthesis to physiological maturity. The data on grain traits was recorded from three main spikes harvested and threshed separately. GFR was estimated as the ratio between single grain weight (SGW) per RILs and GFD.

DNA extraction

DNA was isolated by N-cetyl-N, N, N-trimethyl ammonium bromide

(CTAB) method (Saghai-Maroof et al., 1984). Leaves were detached when the seedlings reached the 5 to 6-leaf stages and were frozen immediately in liquid nitrogen and utilized for DNA isolation. A sample of 200 to 400 mg of leaf tissues per plant was powdered by grinding in liquid nitrogen and incubated in CTAB buffer (200 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB, 1% PVP) at 65°C for 40 min. The extract was washed with chloroform/isoamyl alcohol (24: 1, v/v), and the emulsion was centrifuged at 12000 rpm for 15 min. The supernatant was precipitated with equal amount of isopropanol, and pellet was washed with 70% ethanol, and dissolved in 1 x Tris-ethlenediaminetetracetic acid (EDTA). The DNA quality was checked on 0.8% agarose gel.

PCR amplification

The PCR was performed in a volume of 25 µl containing 18.17 µl Nuclease free water 2.5 µl 10x (final concentration 1x) buffer with $MgCl_2$ mixed, 2 µl dNTP (200 µM of each dNTP) , 1 µl Primer (F+R), (0.2 µM of each primer) , 0.33 µl Taq Polymerase (1.0 unit) (Bangalore Genie, India, now merge in Merck) and 1 µl of template DNA. The reaction mixture was run on a Thermocycler (BioRad, USA), by procedure of Roder et al. (1998) with minor modifications. The PCR cycle included an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 50, 55 or 60°C (depending on the individual microsatellite primer) for 1 min; and extension at 72°C for 1 min followed by a 6 min final extension at 72°C. Amplification products were resolved by electrophoresis on 3% agarose gels (Genie, now merge with Merck, Bangalore, India) at 80 V for 45 min, visualized by ethidium bromide staining, and gel photograph taken by Geldoc system (Syngene Ltd., USA).

Data and linkage analysis

The phenotypic data was analysed using CROPSTAT (IRRI) software. The association between molecular markers (simple sequence repeat, SSR) and the difference in grain filling rate as indicator for heat tolerance genes was assessed with simple regression analysis. Magnitude of the marker associated with phenotypic effect was described by the coefficient of determination (R^2) which represented the fraction of variance explained by the polymorphism of the marker.

RESULTS

Microsatellite marker analysis

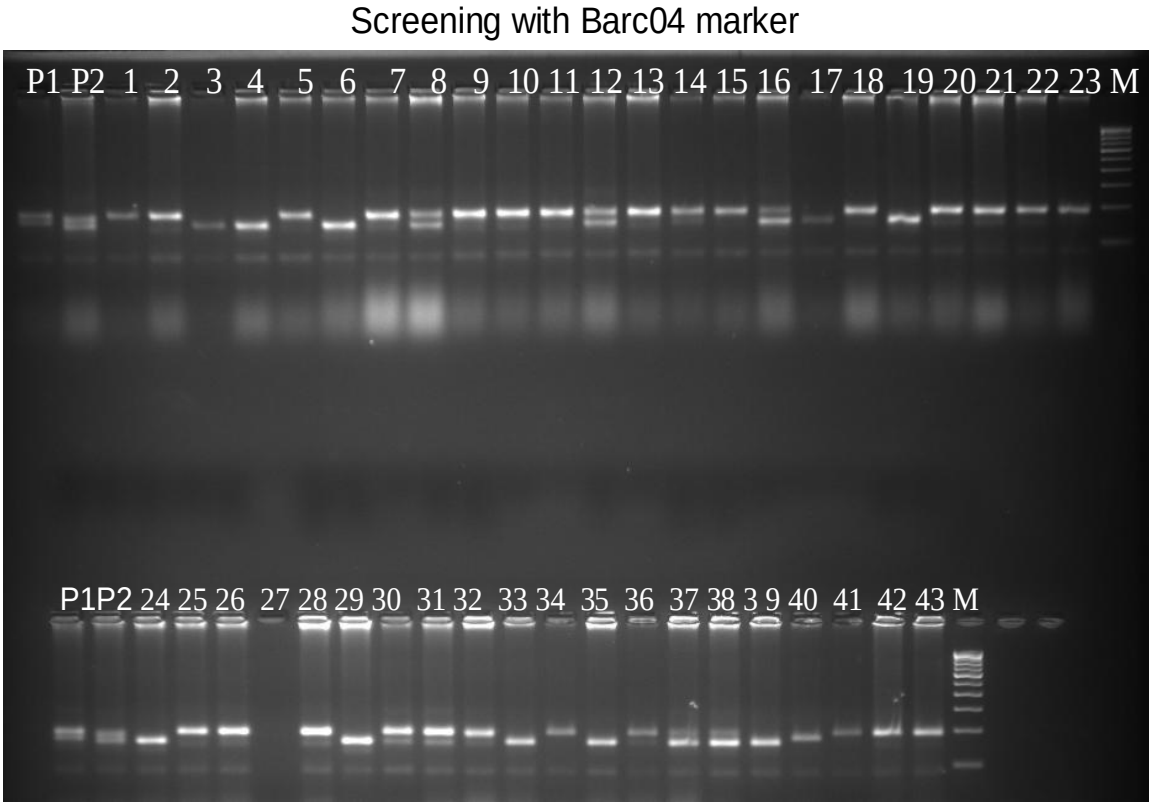
Raj 4014 and WH 730 were screened with different SSR markers such as GWM (Roder et al., 1998), WMC, GDM, BARC, CFD, CFA, PSP among others. Out of 300 SSR markers tested, 15% were found polymorphic. These polymorphic markers were utilized for genotyping a subset of RILs that had clear contrasting variation for dGFR. To check for potential co-segregation of DNA fragments and heat tolerant phenotypes, simple regression analysis was carried out in order to confirm an association between the markers and the grain filling rate as indicator for heat tolerance. Out of the 35 markers tested, relationship between the two markers Xbarc04 and Xgwm314 and the phenotypes of RILs got established which were highly significant. The coefficient of determination (R^2) was recorded 0.10 and 0.06, respectively. This indicates that the two markers were associated with the differences in grain filling (dGFR) rate as indicator for heat tolerance. Xbarc04 (Figure 1) and Xgwm314 have their locus position on chromosomes 5B and 3D, respectively (Table 2).

Field data analysis

Average temperatures during post heading period under timely and late sown conditions of crop seasons 2009-2010 and 2010-2011 was higher by 1.1 and 3.1°C, respectively, under late sown conditions (Figure 2). Analysis of variance revealed that the genotypes as well as conditions of sowing differed significantly for grain filling rate (Table 3). The dGFR in parental lines under late sown conditions was 0.168 in RAJ 4014 and -0.258 in WH 730 and it ranged from -0.549 to 0.430 in RILs. The distribution of dGFR in RILs showed normal curve. About 23% progenies (26 RILs) had significant difference in GFR under late sown condition whereas 83 RILs had higher GFR under stress conditions.

DISCUSSION

In heat stress environments, genotypes' sensitivity is generally expressed by difference in grain yield. Additionally, the temperature X genotype interactions also suggests the occurrence of genetic variability for adaptability to high temperature (Hunt et al., 1991; Dias and Lidon, 2009). The GFR is the most important parameters associated with grain development, which determine the productivity of wheat genotypes (Yang and Zhang, 2006). In general, the process of grain filling is regulated by both GFR and GFD (Gebeyehou et al., 1982; Wang et al., 2009); however, their relative contribution remains debatable. Mashiringwani and Schweppenhauser (1992) reported that genotypic differences in grain yield of wheat were due to differences in GFR. It indicates that genetic differences in final grain weight were related to differences in GFR rather than GFD as GFD and GFR is also influenced by rise in temperature. However, GFR is generally accelerated to compensate for reduction in GFD. The two parents, RAJ 4014 and WH 730 differed in GFR under high temperature stress conditions. For crop season 2009 to 2010, the distribution of dGFR (Figure 3) in RILs showed normal curve. About 23% progenies (26 RILs) had significant difference in GFR under late sown condition whereas 83 RILs had higher GFR under stress conditions. During crop season 2010 to 2011, about 20% progenies had significant difference in GFR under late sown conditions (Figure 4). The crop season 2010 to 11 was warmer as compared to 2009 to 2010. The quantitative frequency distribution of dGFR suggested that this parameter could be used as a selection criterion.



P1=WH730,P2=Raj4014,1=RIL1,2=RIL2,3=RIL5,4=RIL6,5=RIL7,6=RIL9,7=RIL16,8=RIL17,9=RIL18,10=RIL20,11=RIL 22,12=RIL28,13=RIL30,14=RIL39,15=RIL42,16=RIL44,17=RIL46,18=RIL50,19=RIL52,20=RIL54,21=RIL55,22=RIL61,23=RIL62,24=RIL64,25=RIL67,26=RIL71,27=RIL72,28=RIL74,29=RIL92,30=RIL96,31=RIL97,32=RIL101,33=RIL110,34=RIL112,35=RIL114,36=RIL116,37=RIL118,38=RIL120,39=RIL133,40=RIL134,41=RIL136,42=RIL139,43=RIL143, M=100bp.Ladder

Figure 1. Screening of RILs by Xbarc04 marker. M, 100bp; P1, WH730; P2, Raj4014, RILs, 1-43.

Table 2. Primer sequences of both markers.

Marker	Primer sequence	Annealing temperature (ºC)	Chromosome number
Xbarc 04	GCGTGTTTGTGTCTGCGTTCTA CACCACACATGCCACCTTCTTT	55	5B
Xgwm 314	AGGAGCTCCTCTGTGCCAC TTCGGGACTCTCTTCCCTG	60	3D

that this parameter could be used as a selection criterion. This finding underlines the potential value of dGFR as a selection parameter for identifying superior wheat lines under heat stress conditions. Regression analysis revealed that the two markers Xbarc04 (R^2=0.10*)*, and Xgwm314 (R^2=0.06*)* could explain the variation in the phenotypes of RILs. This indicates that these two markers are associated with the dGFR as indicator for heat tolerance. The R^2 values suggested that the Xbarc04 and Xgwm314 *a*ccounted for 10 and 6% of phenotypic variation in heat tolerance in the RILs population, respectively. The T-test value for Xbarc04 was 5.58 and correlation was -0.32 and T-test value for Xgwm314 was 5.28 and correlation was 0.24. It is suggested that these two markers may be useful in marker-assisted selection for improving productivity of Indian wheat under high temperature environment. However, these markers alone cannot be the exclusive selection criterion for heat tolerance in wheat breeding. It may be valuable as a supplemental criterion in the final breeding stages or as a selection tool to reduce a large population into the most likely heat tolerant core at the

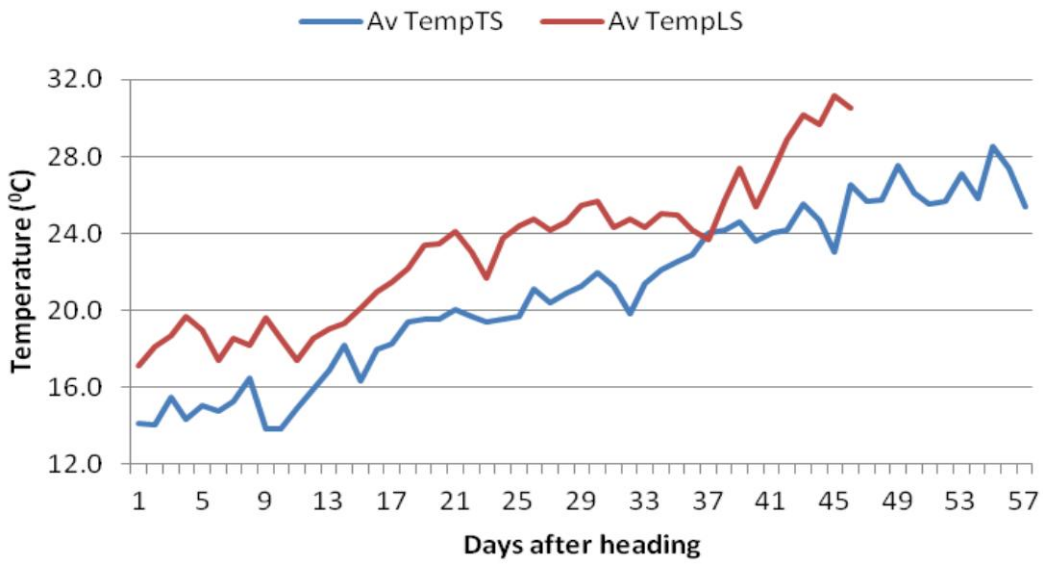


Figure 2. Average temperatures (Av Temp) during post heading period under timely sown (TS) and late sown (LS) conditions of crop seasons 2009-2010 and 2010-2011.

Table 3. Analysis of variance for grain filling rate.

Source of variation	DF	Mean squares	
		2009- 2010	2010- 2011
Genotypes	114	0.0849**	0.126**
Replication	1	0.018	0.053
Time of sowing	1	2.041**	0.532**
Time of sowing* Genotype	114	0.0368**	0.055**
Residual	228	0.023	0.018
Total (corrected)	459	0.046	0.055

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

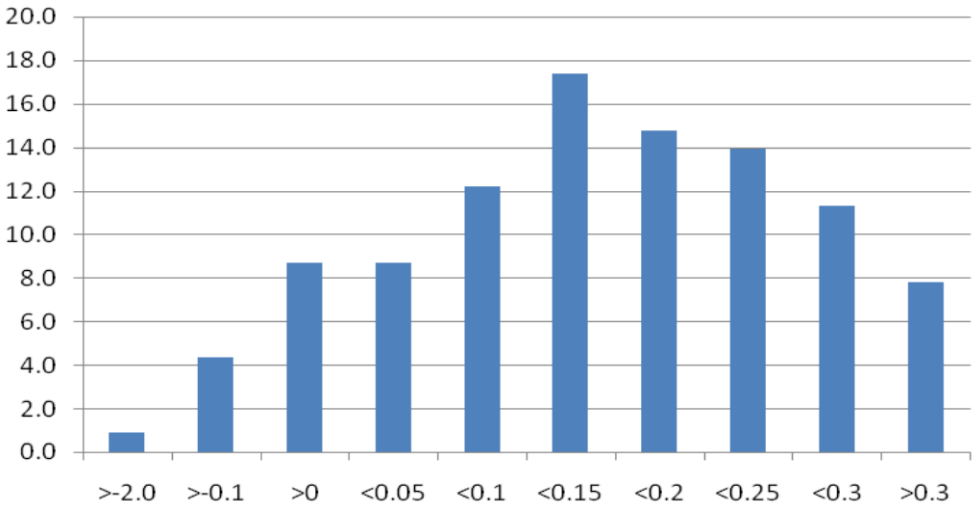


Figure 3. Genetic variation in RILs of RAJ 4014/WH 730 for dGFR in crop season 2009-2010 (dGFR= GFRTs-GFRLs).

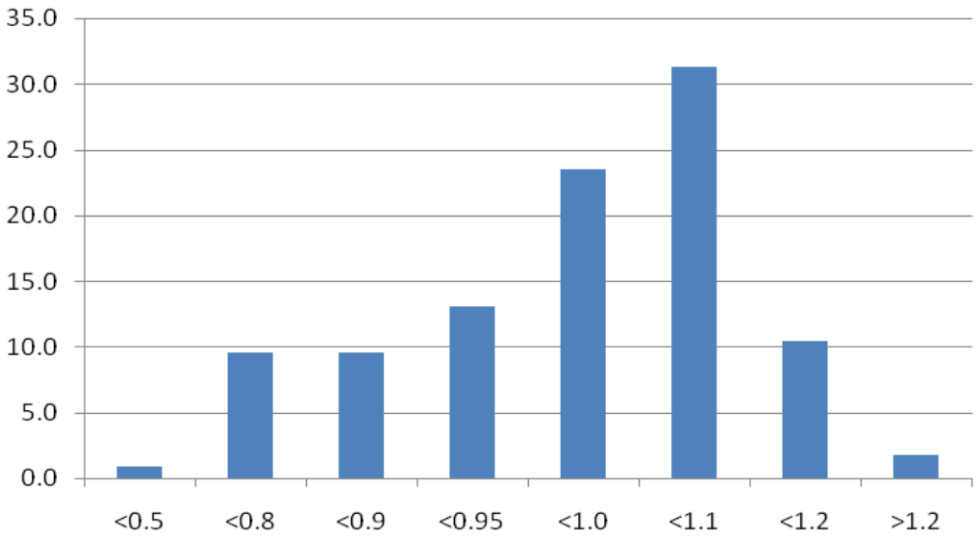


Figure 4. Genetic variation in RILs of RAJ 4014/WH 730 for dGFR in crop season 2010-2011 (dGFR= GFRTs-GFRLs).

early stages of the breeding program. Further, to expand the application of these markers additional investigations are essential with different set of mapping population under different heat stress environments.

In future, further characterization of gene(s) controlling GFR by fine mapping will provide breeders not only a tool in form of markers for selecting genotypes there by giving opportunity to deal with heat stress, but also tailoring wheat genotypes suitable for late planting. With their enhanced capacity to tolerate rise in temperatures, these genotypes can facilitate intensive farming through a range of cropping sequences.

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