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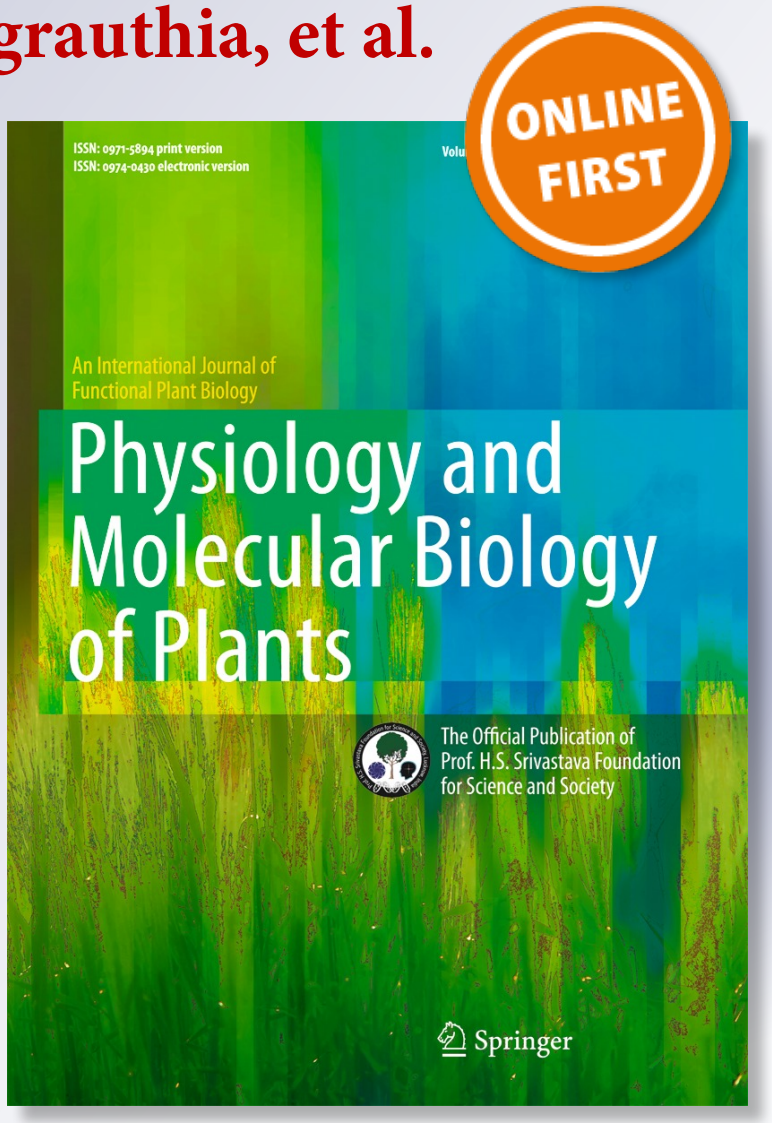

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RESEARCH ARTICLE

Field level evaluation of rice introgression lines for heat tolerance and validation of markers linked to spikelet fertility

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Abstract Rice lines derived from wild species and mutants can serve as a good resource for favorable alleles for heat tolerance. In all, 48 stable lines including 17 KMR3/*O. rufipogon* introgression lines (KMR3 ILs), 15 Swarna/*O. nivara* ILs (Swarna ILs) along with their parents, Nagina 22 (N22) and its 4 EMS induced mutants and 7 varieties were evaluated for heat tolerance under irrigated conditions under field in two seasons, wet season 2012 using poly cover house method and dry season 2013 using late sown method. Spikelet fertility (SF), yield per plant (YP) and heat susceptibility index (HSI) for these two traits were considered as criteria to assess heat tolerance compared to control. Four KMR3 ILs and eight Swarna ILs were identified as heat tolerant based on SF and YP and their HSIs in both wet and dry seasons. S-65 and S-70 showed low SF and high YP consistently in response to heat in both seasons. We provide evidence that SF alone may not be the best criterion to assess heat tolerance and including YP is important as lines with low SF but high YP and *vice versa* were identified under heat stress. Out of 49 SSR markers linked to spikelet fertility, 18 were validated for five traits. RM229 in wet season and RM430 and RM210 in dry season were significantly associated with both SF and its HSI under heat stress. RM430 was also significantly associated with both YP and its HSI in dry season. Thirty two candidate genes were identified close to nine markers associated with traits under heat stress.

Keywords Heat stress · Wild rice · Introgression lines · Spikelet fertility · Heat susceptibility index · Association mapping

Abbreviations

IL	Introgression line
QTL	Quantitative trait loci
SSR	Simple sequence repeat
SF	Spikelet fertility
YP	Yield per plant
HSI	Heat susceptibility index
AHDD	Accumulated heat degree days
LD	Linkage disequilibrium
GLM	General linear model
MLM	Mixed linear model

Introduction

The global air mean temperature is expected to be 2 to 4 °C higher than now by the year 2100 largely due to emission of green house gases. Crops grown in future environment are anticipated to face extreme temperatures of short duration at higher frequency than now (IPCC:Intergovernmental panel on climate change 2013). Exposure of crops to these extreme higher temperatures during sensitive stages of development reduces yield significantly. Rice is a major staple crop growing in irrigated systems in South Asia where the temperature exceeds critical threshold level (Nakagawa et al. 2002) and its productivity is decreased by increasing seasonal temperature (Welch et al. 2010). The sensitivity to heat stress varies with species and cultivars, the duration and intensity of heat stress, the phenological stage of the plants, and several other

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environmental factors. It is very important to understand crop response to high temperatures to minimize the adverse effect.

Heat tolerance is a complex trait involving different genetic components, numerous morpho-physiological and biochemical mechanisms, and their interactions both spatially and temporally. Exploring the thermo tolerance diversity in rice plants will provide useful information for the improvement of traits to cope with the projected climate warming scenarios in the future (IPCC: Intergovernmental panel on climate change 2007). The impact of heat stress on rice yield depends on the genotype, the growth stage at which heat stress is received and the period of the stress (Liao et al. 2014; Poli et al. 2013; Zhou et al. 2012; Ye et al. 2011; Jagadish et al. 2010a; Prasad et al. 2006; Morita et al. 2005). In general, when rice plants are exposed to temperature greater than 35 °C for more than one hour at anthesis, it results in increased sterility (Jagadish et al. 2007). High temperature stress caused abnormal anther dehiscence and poor pollen germination which in turn increased spikelet sterility (Matsui and Omasa 2002; Jagadish et al. 2010a). Heat stress during flowering stage negatively impacts both percent seed set and total grain yield (Bui et al. 2014; Morita et al. 2005; Peng et al. 2004). The critical physiological parameter "spikelet with exerted anthers but with no ovule enlargement" was considered as the most sensitive stage with exposure to heat stress at important reproductive stages (Shi et al. 2015b). Later, the milky stage (6–16 d after heading) during grain filling is also a period sensitive to high temperature as it prevents the photosynthate transportation to grain, shortens the effective grain filling stage and thus reduces grain weight (Liao et al. 2011; Shi et al. 2015b).

However, different genotypes of rice cope differently with heat stress. Experiments on heat stress under field conditions are very few. Geographic origin of rice varieties was not clearly related to the degree of tolerance or susceptibility to heat stress because both conditions were observed in cultivars from each target region (Shi et al. 2015b). N22 seedlings showed higher basal thermo tolerance level than Nipponbare but results were opposite when the long term acquired thermo tolerance level was considered. Genetic variation in the ability of rice to maintain spikelet fertility and seed set under heat stress allows selection of genotypes with increased tolerance to heat stress. QTL mapping and gene expression studies for heat tolerance have been carried out in rice at different stages such as booting, flowering and grain filling stage (Bui et al. 2014; Wei et al. 2013; Liao et al. 2014; 2012, 2011; Poli et al. 2013; Xue et al. 2012; Xiao et al. 2011; Ye et al. 2011; Jagadish et al. 2010a; Zhang et al. 2008; Chen et al. 2008; Zhao et al. 2006). QTLs for heat tolerance were mapped on all chromosomes except chromosome 7 but fine mapping of these QTLs has not been reported. Identification of QTLs for heat tolerance may be limited by low marker coverage and small QTL effect (Steele et al. 2006; Jena and Mackill 2008). Association mapping is another approach used for identifying genes controlling important traits (Borba et al. 2010). A significant number of QTL for important traits have been found by genome-wide association studies (GWAS) in rice (Courtois et al. 2013; Samuel et al. 2010; Zhao et al. 2007). Fine mapping of major effect QTL can then help in short listing candidate genes. Nine marker-trait associations between polymorphic SSR markers and three agronomic traits *viz.*, panicle length, hundred grain weight and yield per plant were reported after association analysis in Tamil Nadu landraces together with *indica* and *japonica* cultivars showed (Vannirajan et al. 2012). Most of these studies were carried out in controlled conditions, where plants were subjected to a few hours of heat stress at critical stages and returned to normal temperature. Plants often have the ability to acclimatize to stress if it persists for long at sub-optimal levels. The variability in acclimation also needs to be explored for which field level screening is required in appropriate locations. The effect of short duration stress given only at reproductive stage may be quite different than a long duration stress where plants can acclimatize to high temperature during vegetative stage itself. Hyderabad is a good location for studies on heat stress in low humidity conditions (Poli et al. 2013).

The wild species, *Oryza rufipogon* and *O. nivara* are the progenitors of *O. sativa* and constitute an important gene pool for improvement of rice. Molecular marker mapping studies in rice have shown that wild species can contribute genes for improving composite traits such as yield and abiotic stress tolerance, in spite of their poor phenotype (Swamy and Sarla 2008: Nevo and Chen 2010; Placido et al. 2013). There are also some rice cultivars and landraces that show significant variation in physiological and morphological characters. We used 48 rice genotypes including two sets of stable introgression lines derived from elite variety x wild species crosses. These were KMR3 ILs and Swarna ILs (Advanced BILs developed using wild species *O. rufipogon* and *O. nivara* respectively) along with their parents, and N22 and its mutants and improved cultivars. These genotypes are genetically diverse and are being used in breeding programs and for QTL mapping because of their wide range of agronomic attributes. The main aims of present research were to identify (1) heat tolerant lines among wild introgression lines, landraces, varieties and mutants using two field methods in two seasons and (2) significant marker trait associations for both spikelet fertility and grain yield.

Materials and methods

A total of 48 stable lines consisting of five sets were used as experimental material. The details are as follows: Set1- - KMR3 a restorer line and 17 KMR3 × *O. rufipogon* introgression lines (KMR3 ILs), Set2- Swarna, a popular cultivar and 15 Swarna × *O. nivara* ILs (Swarna ILs), Set3- Nagina 22

(N22) a heat tolerant aus variety and its 4 EMS induced mutant lines NH219, NH363, NH686, NH787 and Set 4 and 5 included 9 wild rice/landraces/improved varieties *viz.*, *O. rufipogon* (WR120), *O. nivara* (IRGC 81848 and IRGC 818132) Bala, IR64, Azucena, Moroberekan, Nipponbore, Tequing and Vandana. The details of the lines are given in Supplementary Table 1.

Phenotyping

Heat stress experiments were carried out using 48 genotypes at IIRR field (latitude and longitude: 17° 22′31″ N and 78° 28′ 27″E) during wet season 2012 using poly cover house method and dry season 2013 using late sown method (flowering coinciding with high temperature) for heat stress. Controls were sown normally in each year i.e. without poly cover in 2012 and sown in normal sowing time in 2013. Essentially, the experimental plants in poly cover house method in wet season 2012 were grown as in control for the entire vegetative stage of growth and experienced elevated temperature only from the end of vegetative stage till harvest but the experimental plants in late sown method in dry season 2013 experienced slow increase of temperature during vegetative stage itself.

Poly cover house method during wet season 2012

For this study, plants were grown in 6 replications of 2 lines of each genotype and 22 plants per line with spacing of 20 cm × 20 cm. When the plants attained booting stage, plants in 3 replications were covered with polythene sheet of 1 mm thickness (only 8–10 % cut in radiation) over metal rods to provide heat stress till maturity as shown in Supplementary Figure 1 (Poli et al. (2013). The minimum and maximum temperature and relative humidity (RH) inside and outside polycover house were recorded during this period using thermo hygrometer and photometer (N.S. Dimple Thermometers, Delhi, India). The ambient temperature in normal conditions was recorded as ≤33 °C, ≥ 24.5 °C, and average 30 °C during day time. The RH under ambient normal conditions was ≥30 %, ≤ 97 %, and average 87 %. The temperature inside polythene sheet cover was ≥30.2 °C, ≤ 48 °C, and average 44.3 °C during day time. The details of temperature recorded during the experiment are shown in Fig. 1. The RH was generally 5–8 % higher inside the poly cover house than ambient conditions. Normal irrigation and fertilizer conditions were followed as per recommended agronomic packages and practices in both control (uncovered) and experimental plots in field.

Late sown method during dry season 2013

Field trials were conducted during dry season 2013 by sowing at two different times at an interval of 21 days. Normal sowing was done in December (as control condition) and late sowing in January (as heat stress condition) (Supplementary Figure 1). The main aim of this field trial of sowing in different dates in the same location was to maintain all parameters of soil and environment nearly same except high temperature experienced by the late sown crop. The experiment was carried out in 3 replications of 2 lines of each genotype and 22 plants per line with spacing of 20 cm × 20 cm. Field was maintained as per recommended agronomic packages and practices. Daily mean maximum and mean minimum temperatures and relative humidity were recorded during the entire period of experiment using the weather station at DRR field (Fig. 1). The day temperature during flowering time in normal sowing method was recorded as ≤38.9 °C, ≥ 37.6 °C, and average 35.7 °C where as in late sown method it was ≤42.33 °C, ≥ 40.0 °C, average 37.6 °C. The relative humidity recorded during flowering time in both cases was not significantly different.

Days to flowering, accumulated heat degree days (AHDD) till flowering, % spikelet fertility and yield per plant were recorded under both control and high temperature conditions in both the above experiments. Accumulated heat degree days till flowering (AHDD), experienced by each genotype in all treatments were calculated by base temperature cut-off of 10 °C method (d'Alpoim Guedes et al. 2015; Shi et al. 2015a). Spikelet fertility was calculated as the ratio of partially and fully filled spikelets to total number of spikelets per panicle. Spikelet fertility% for each genotype was calculated by evaluating 3 panicles each from 3 plants for each replication. Heat susceptibility index (HSI) for % spikelet fertility and yield per plant were calculated by using formula:

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

where Yh and Y are the means of a phenotype for each genotype under heat stressed and control conditions respectively and Xh and X are the means of that phenotype of the population as a whole under heat stressed and control conditions respectively. Genotypes with HSI < 0.6 were considered as heat tolerant lines (Fischer and Maurer 1978).

Genotyping

DNA was isolated from the leaves of above experimental material using CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer and genotyping was done using 49 SSR markers. These markers were selected based on earlier studies where they were either reported to be linked or were close to QTLs for spikelet fertility or pollen fertility under high temperature stress. Our interest was to see if any of these were also associated with yield since yield per plant was not reported in the previous studies. The list of markers used, QTLs linked or close to these markers and references are given in Supplementary Table 2. The

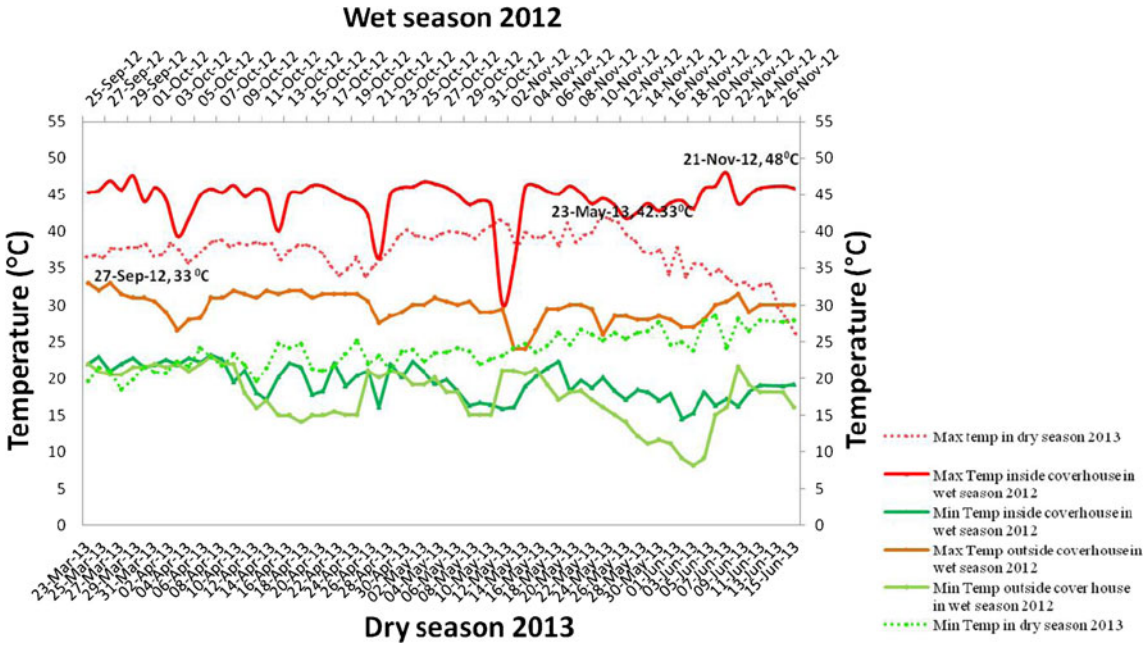

Fig. 1 Maximum and minimum temperature (°C) recorded from booting stage till maturity in field during wet season 2012 and dry season 2013

primer sequence, annealing temperature and repeat motifs etc. for all these markers are available at http://www.gramene.org/markers/microsat. Alleles of each SSR locus were scored as presence (1) or absence (0) of the respective fragment size. Bands that were diffuse or too difficult to score were considered as missing data ('-'). Dendrogram was constructed based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method based on genotyping data. The informativeness of a marker was measured by calculating Polymorphism information content (PIC) using formula.

$PIC = 1-\sum p^2_{ij}$ where p_{ij} is the frequency of j^{th} SSR allele for the marker i.

Population structure

A total 49 SSR polymorphic markers data of 48 genotypes belong to all five sets as described earlier and their phenotypic data under control and high temperature conditions from wet season 2012 and dry season 2013 were used to study the marker-trait association. The Q matrix (population structure) model was considered for population structureto avoid possible false positive associations. The genetic structure prediction and clustering of the population was done with the Bayesian model based software program STRUCTURE v2.3.4 (Pritchard et al. 2000). The number of groups (*k*) was tested between 1 and 10 by running STRUCTURE with the admixture model, a burn-in period of 10,000 and Markov Chain Monte Carlo repetitions of 10,000 (Evanno et al. 2005). The *k* value at which the posterior probability (Ln P (D)) plateaus as described in Casa et al. (2008) was used for association analysis. The correct estimation of *k* was provided by Log probability data [LnP (*D*)] as function of *k* (number of groups) from the STRUCTURE run (Evanno et al. 2005) which was based on the rate of change in the log probability of data between successive *k* values. Based on the correct k value, each genotype was assigned to a group for which its membership value (Q value) was >0.5 (Pritchard et al. 2000) and the population structure matrix was generated for further analysis.

Association analysis

Linkage disequilibrium (LD) was estimated for each pair of SSR loci across all 48 genotypes using TASSEL v 3.0.91(Bradbury et al. 2007). LD was calculated using D' and r^2 estimates and significance of D' for each SSR pair was determined by 10,000 permutations. The association mapping based on these models was performed with the software TASSEL v3.0.91 (Pritchard et al. 2000). The 5 % 'minor alleles' filtered SSR datasets were used for this association mapping models. Two statistical models *viz.*, general linear model (GLM) and mixed linear model (MLM) were used to account for population structure and genetic relatedness. The Q matrix (created by programme STRUCTURE) was considered and the ancestry coefficients estimates were used as covariates in GLM model. In case of MLM model, both Q matrix (created by programme STRUCTURE) and the kinship matrix (K) (generated in TASSEL itself) were considered. K matrix of the genetic relation among pair-wise genotypes was used. Markers were regarded as significantly associated with traits on the basis of their adjusted *P* value ($-\text{Log } P \geq 2.00$; $P \leq 0.05$) for GLM and MLM.

Candidate gene analysis

Physical positions of the markers associated with traits using GLM and MLM methods in heat stress conditions were obtained from Gramene data base (http://www.gramene.org/). All genes 1 Mb upstream and 1 Mb downstream regions from these marker positions were noted from RAPDB (http://rapdb.dna.affrc.go.jp/) to identify putative candidate genes for heat tolerance.

Results

Phenotypic performance

Descriptive statistics for flowering time, accumulated heat degree days till flowering (AHDD), % spikelet fertility and yield per plant, HSI of both spikelet fertility and yield under normal conditions and cover house/late sown conditions in wet season 2012 and dry season 2013 are shown in Table 1. Absolute mean values of all the above traits for each genotype are given in Supplementary Table 3. All lines flowered earlier in high temperature when compared with that of ambient conditions in wet season but in dry season, the average flowering time of all genotypes in late sown method (127 days) was more than in control (normal sowing date). N22, Bala and Vandana were early flowering genotypes as they flowered in 85–104 days. Difference in mean AHDD was highly significant between control and heat stress conditions in both the seasons. During wet 2012 season, under heat stress condition, the mean AHDD was 5 % more compared to that of control condition where as during dry 2013 season, it was 15 %. In spite of variation in flowering time between control and heat stress conditions, almost all genotypes received more AHDD under heat stress conditions in both wet and dry seasons. The genotypes were exposed to higher AHDD (~ 419 AHDD more) in dry season than in wet season under heat stress conditions. Percent spikelet fertility was reduced in all genotypes under high temperature conditions in both the seasons except in K-13-5 and K-198 in poly cover house and in K-13-7, K-103, K-458, SM686, Bala, and Tequing in late sown conditions. Yield per plant was also reduced in all genotypes under high temperature conditions in both the seasons except in S-65, S-70 and K-50 in poly cover house and in eight Swarna ILs (S-10-3, S-14-3, S-65, S-70, S-75, S-148, S-230 and S-248) and four KMR3 ILs (K-13-5, K-13-7, K-16-3 and K-377-24) in late sown conditions. However, the increase in yield per plant was significant only in two ILs (S-65 and K-377-24) by 30 % and 25 % in late sown conditions in dry season. K-377-24 showed maximum yield of 32.5 g/plant and S-65 showed 13 g/plant in dry season under heat stress conditions. Three Swarna ILs *viz.*, S-24, S-230 and S-250 showed significant increase in both spikelet fertility and yield per plant over their respective control parents under heat stress conditions in both wet and dry seasons whereas S-14-3 and S-248 in dry season only and S-13-5 and K-198 in wet season only. Figure 2 shows the regression analysis between spikelet fertility and yield per plant along with regression equations and r^2 values. Pearson correlation between traits across two treatments and two seasons is given in Supplementary Table 4. Flowering time and accumulated heat degree days were highly correlated under both normal and heat stress conditions in both wet 2012 and dry 2013 seasons. Yield/plant in normal conditions in wet season 2012 was correlated with yield/plant in dry season normal conditions. Yield/plant in cover house wet season 2012 was correlated with yield/plant in late sown dry season 2013. These results indicate that either of the two methods can be used for screening in any season. In wet season 2012, % spikelet fertility in cover house was weakly correlated with yield/plant but in dry season late sown conditions 2013, % spikelet fertility was strongly correlated with yield/plant in. Only in dry season 2013, yield/plant in normal sowing was correlated with yield/plant in late sowing conditions. This was true of % spikelet fertility also but yield and spikelet fertility were not correlated in normal conditions. Yield/plant in wet season 2012 normal conditions was strongly correlated with both yield/plant and % spikelet fertility in dry season late sown conditions. Significant correlation was observed between spikelet fertility and yield per plant only under heat stress conditions in both the methods of poly cover ($r^2 = 0.13$; $p < 0.05$; Fig. 2(b)) and late sown ($r^2 = 0.34$; $p < 0.001$; Fig. 2(d)) but there was no such correlation observed in control conditions in both wet and dry seasons.

Besides absolute values for % spikelet fertility and yield per plant, their heat susceptibility indices (HSI) were also considered for identifying heat tolerant and susceptible genotypes. During wet season, seven KMR3 ILs and three Swarna ILs and during dry season, eight KMR3 ILs along with KMR3, nine Swarna ILs, four N22 and its four mutants, Bala and Tequing showed <0.6 HSI for spikelet fertility. Swarna IL S-148 was considered as most heat susceptible in wet season while S-166-2 and S-166-30 in dry season as their HSI of spikelet fertility was >3.5. HSI for yield per plant was <0.6 in three KMR3 ILs and four Swarna ILs during wet season and six KMR3 ILs along with KMR3 and eleven Swarna ILs during dry season. Out of 48 lines, four KMR3 ILs (K-13-5, K-16-3, K-377-24 and K-458) along with KMR3 and four Swarna ILs (S-10-3, S-14-3, S-24 and S-250) showed <0.6 value for three HSIs out of four HSIs for spikelet fertility and yield per plant.

Genotypic diversity using SSR markers

In all, 49 SSR markers, selected based on earlier reports of markers linked or close to QTLs for pollen fertility or spikelet fertility under high temperature stress, distributed across all 12

Table 1 Phenotypic Performance of 48 genotypes under normal conditions and cover house/late sown method during wet season 2012 and dry season 2013

	Normal Conditions				Cover house/late sown conditions			
Season/trait	Minimum	Maximum	Mean	SD	Minimum	Maximum	Mean	SD
Wet season 2012								
Flowering time (days)	85.00	116.00	110.73	6.44	85.00	113.00	104.88	5.89
Accumulated heat degree days till flowering	1387	1845	1768	92	1401	2036	1854	130
Spikelet fertility (%)	64.79	91.97	82.37	6.34	17.58	89.77	61.95	16.78
Yield per plant (g)	8.13	33.66	19.71	6.32	4.27	21.12	10.74	3.75
HSI for spikelet fertility	−0.42	3.21	1.00	0.78				
HSI for yield per plant	−0.69	1.78	0.89	0.59				
Dry season 2013								
Flowering time (days)	71.00	150.00	122.18	15.14	93.00	152.00	127.34	16.66
Accumulated heat degree days till flowering	1390	2526	1950	278	1591	2706	2273	310
Spikelet fertility (%)	70.00	97.75	89.05	6.54	20.00	96.16	77.63	16.98
Yield per plant (g)	9.73	32.33	20.75	6.67	4.84	32.52	16.45	5.86
HSI for spikelet fertility	−0.61	5.57	1.04	1.33				
HSI for yield per plant	−2.45	3.41	0.81	1.41				

rice chromosomes except chromosome 7 were used. Chromosome wise marker allele segregation statistics and PIC values are shown in Table 2. A total of 171 alleles were detected with an average of 3.45 alleles per marker. Among the 171 alleles, 54 were rare with less than 5 % frequency among 48 genotypes. There were 2–8 alleles per SSR locus. The frequency of biallelic markers was maximum at 30.6 %, triallelic were 26.5 % and tetrallelic were 24.5 %. The average PIC values ranged from 0.07 (RM348) to 0.96 (RM183). The average PIC value was maximum for markers on chromosome 9 (0.68) and least for markers on chromosome 10 (0.24). Dendrogram, based on SSR marker data, revealed five groups and the dissimilarities ranged from 0.02 to 0.81 (Fig. 3). KMR3 and all 17 KMR3 ILs clustered into two groups where as Swarna and its 15 Swarna ILs except S-148 were in one cluster. All four N22 mutants clustered as one group along with *O. rufipogon*, Bala and IR64. The phylogeny tree revealed that Vandana and Moroberekan appear in the same cluster of Swarna ILs. The aus variety N22, japonica varieties Tequing, Azucena, Nipponbore, wild accessions of *O. nivara*, and Swarna IL S-148 and clustered together though their dissimilarity was more than 0.6.

Population structure

STRUCTURE results exhibited that k value showed flattening of curve at k = 5 indicating that five groups might contain all genotypes with more probability (Fig. 4). All 17 KMR3 ILs

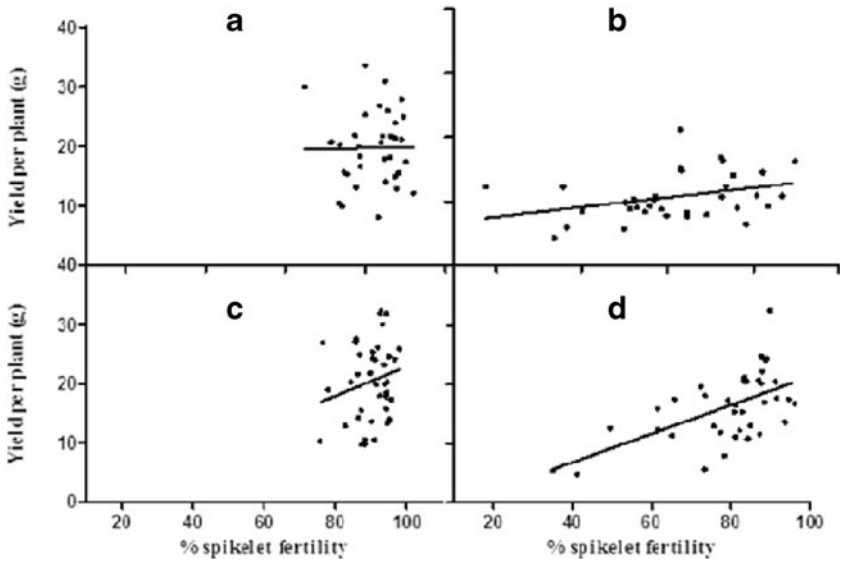

Fig. 2 Relationship between spikelet fertility and yield per plant in (**a**) wet season 2012 control; (**b**) wet season poly cover method; (**c**) dry season 2013 normal sowing; and (**d**) dry season 2013 late sowing. Regression equations: (*a*) $y = 0.01\times + 18.93$; $r^2 = 0.0001$; (*b*) $y = 0.16\times + 12.4$; $r^2 = 0.133$; (c) $y = 0.25\times - 2.06$; $r^2 = 0.041$; (d) $y = 0.24\times - 2.85$; $r^2 = 0.34$

Table 2 Chromosome wise marker allele segregation statistics and polymorphism information content (PIC)

	SSR marker types							Allele number		
Chromosome	Bi	Tri	Tetra	Penta	Hecta	Septa	Octa	Total alleles	Mean	PIC
1	1	1	1				1	17	4.25	0.24
2	1	1	1	1	1	1		27	4.50	0.34
3	1	1	2	1	3			36	4.50	0.41
4	3	5						21	2.63	0.43
5	1		3					14	3.50	0.51
6		2	2					14	3.50	0.58
7								-	-	-
8	3	1		1				14	2.80	0.59
9	1	1						5	2.50	0.62
10	2							4	2.00	0.62
11	2	1	2					15	3.00	0.66
12			1					4	4.00	0.68
Total	15	13	12	3	4	1	1	171	3.34	0.52

along with KMR3 were grouped into one population as Group 1 with a membership proportion of 37.5 % (Table 3) and Group 2 included 6 genotypes *viz*., Bala, IR64, Vandana and three N22 mutants NH219, NH686 and NH787. Three genotypes *viz*., Nipponbore, Azucena and *O. nivara* belonged to Group 3 and six genotypes *viz*., *O. rufipogon*, Moroberekan, Tequing, N22, NH363 and S-148 were in Group 4. Swarna and 14 Swarna ILs were included in Group 5 with membership proportion of 31.25 %. Figure 5 shows the graphical representation of all 48 genotypes across these five groups based on inferred ancestry coefficients. The fixation index (Fst) values of groups varied from 0.15 (Group 4) to 0.83 (Group 1). The pair wise allele frequency divergence values were maximum between group 1 and group 3 (0.36) and minimum between group 3 and Group 4 (0.11).

Association analysis

Linkage disequilibrium was estimated for each SSR locus and in all 315 (31.8 %) pairs showed significant LD ($P < 0.05$) out of 991 pairs. LD decreased with increase in interval distance between pair of SSR markers. The power of LD and number

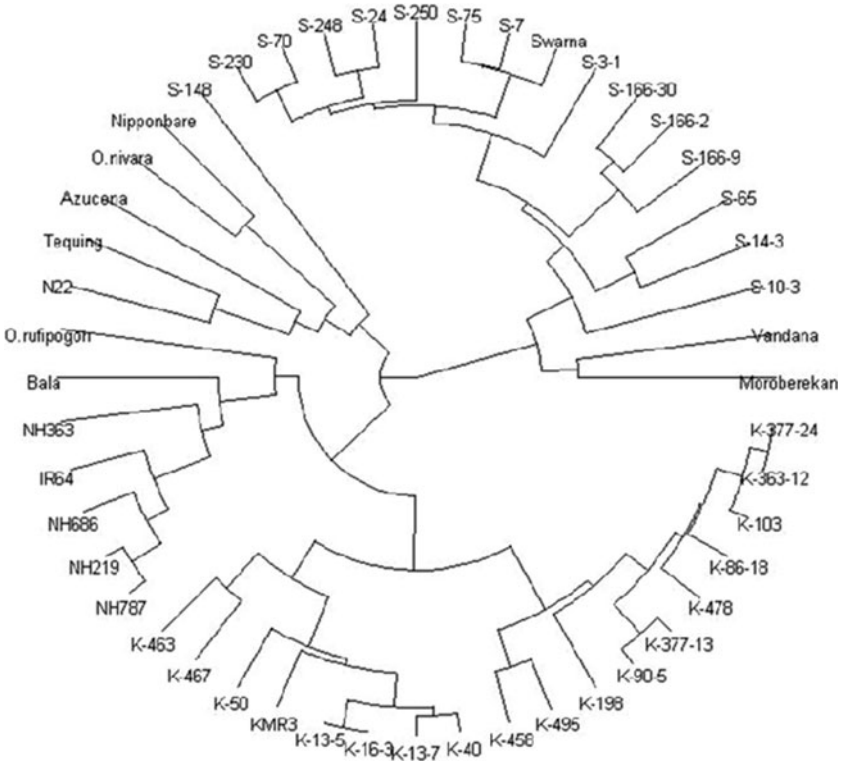

Fig. 3 Dendrogram of 48 rice genotypes by UPGMA method based on SSR marker data

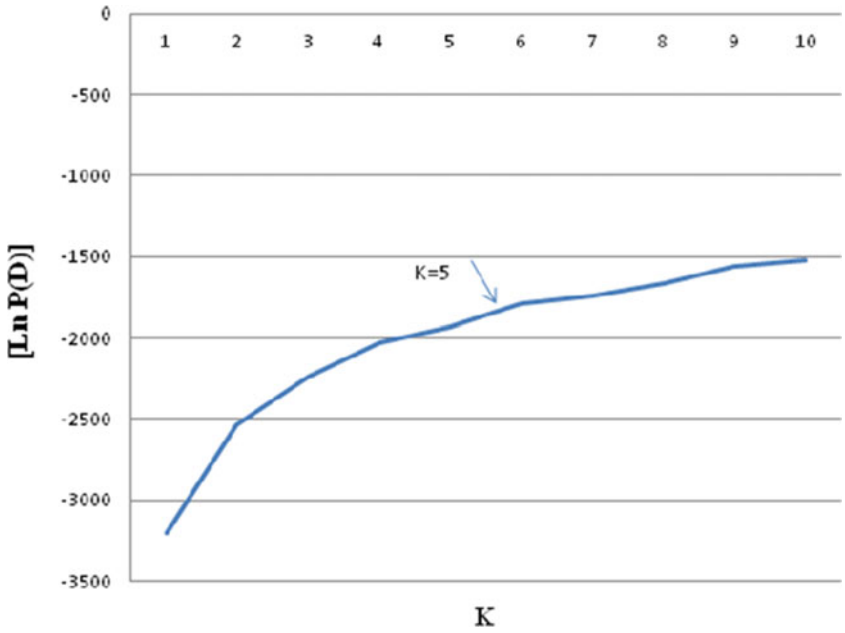

Fig. 4 STRUCTURE results based on SSR marker data. The plateau of the graph at k = 5 indicates the minimum number of groups possible among all 48 genotypes

of marker pairs with significant LD were reduced with increase in distance between marker pairs (Supplementary Figure 2). The results of marker trait association analysis using GLM (Q) and MLM (Q + K) for two phenotyping experiments *viz.*, wet season 2012 (poly cover house method) and dry season 2013 (late sown method) are presented separately in Table 4.

Marker trait association in wet season 2012

Two markers RM250 on chromosome 2 and RM88 on chromosome 8 were significantly associated with days to flowering in normal and poly cover house conditions respectively as detected by GLM. Only one marker, RM229 was associated with spikelet fertility in heat stress treatment with 30 % phenotypic variation observed using both GLM and MLM analysis. RM229 was also associated with HSI for spikelet fertility. Three markers RM250, RM235 and RM570 were significantly associated with yield per plant under normal temperature conditions and explained 16–30 % phenotypic variation. RM274 was associated with HSI for yield per plant using GLM method.

Marker trait association in dry season 2013

GLM analysis revealed significant association of five markers with days to flowering under normal sowing and four markers under late sown conditions (Table 4). RM440 explained maximum phenotypic variation (16 %) for this trait and three markers out of 9 associated markers were significant using both GLM and MLM analysis. Altogether, in both normal and late sown treatments, six markers were significantly associated with spikelet fertility with phenotypic variation of 8 to 25 %. It is significant that two markers RM108 on chromosome 9 and RM167 on chromosome 11 out of these six markers were associated in control and heat stress treatments as well as using both GLM and MLM analysis. Two other markers RM210 on chromosome 8 and RM430 on chromosome 5 were significantly associated with HSI for spikelet fertility. RM430 was the only marker significantly associated with yield per plant and HSI for yield per plant but only in late sown conditions. RM545 was also associated with HSI for yield per plant with 30 % phenotypic variation.

Candidate genes identified 1 Mb upstream and 1 Mb downstream of 9 markers associated with days to flowering, spikelet fertility and yield, all under heat stress conditions are listed in Supplementary Table 5. In all, 32 genes were identified, out of which eight are related to oxidative stress response, seven are defence responsive genes, four are annotated as response to stress and seven are heat shock binding protein genes.

Discussion

In most of the earlier studies spikelet fertility was taken as the main criterion for selecting heat tolerant genotypes but in our study both spikelet fertility and grain yield were considered. Yield/plant in wet season was correlated with yield/plant in dry season. This was true under both normal and stress conditions. Further, there was significant difference in mean accumulated heat degree days between normal and heat stress conditions in both methods. Hence, either of these two methods can be used for screening genotypes for yield under heat stress. Overall results showed positive correlation between spikelet fertility and yield per plant only in heat stress conditions but not in normal conditions. However, there were

Table 3 Population statistics of the estimated groups

Population Group	F_{st}	Heterozygosity	No of Genotypes	Allele frequency divergence					Components
				Group1	Group2	Group3	Group4	Group5	
Group1	0.8256	0.0988	18	-	-	-	-	-	KMR3 and 17 KMR3 Ils
Group2	0.7593	0.1791	6	0.2507	-	-	-	-	Bala, IR64, Vandana and three N22 mutants
Group3	0.3471	0.5022	3	0.3625	0.2955	-	-	-	Nipponbore, Azucena and *O. nivara*
Group4	0.1462	0.5461	6	0.2302	0.1889	0.1134	-	-	*O. rufipogon*, Moroberekan, Tequing, N22, SM363 and S-148
Group5	0.5401	0.2558	15	0.3123	0.2288	0.2075	0.131	-	Swarna and 14 Swarna ILs

Fst Fixation index

few genotypes (S-65 and KMR3–377-24) which did not show significant difference in spikelet fertility between control and heat stress conditions but exhibited significant increase in yield per plant in heat stress. As the ultimate aim of crop production is increased grain yield, these two lines could be considered as heat tolerant lines even though their spikelet fertility decreased in heat stress. If only spikelet fertility was considered these two lines would not have been selected as heat tolerant genotypes. Increased thousand grain weight and increased number of productive tillers per plant may contribute to more yield in these lines under heat stress conditions. The grain size is usually conservative but is mainly increased by increase in assimilates and nitrogen availability to floral parts under heat stress (Sadras 2007).

Based on significant differences between means in control *vs.* heat stress treatments and also over their control parents for both spikelet fertility and yield per plant and their HSIs in both wet and dry seasons, four KMR3 ILs (K-13-5, K-16-3, K-377-24, and K-458) and eight Swarna ILs (S-10-3, S-14-3, S-24, S-65, S-70, S-230, S-248 and S-250) were identified as heat tolerant genotypes. These results indicate that ILs of both Swarna and KMR3 are useful genetic resource for development of heat tolerant lines. Introgression lines of Swarna derived from *O. nivara* accessions have been evaluated for yield, grain quality, and growth in aerobic and low P conditions in the field (Swamy et al. 2011). It may be noted that S-248 was released as DRR Dhan 40 recently for three rice growing states (Maharashtra, Tamilnadu and West Bengal) in India. KMR3 ILs show significant increase in grain yield and the associated traits were early flowering, high number of tillers, panicles, grains, panicle weight and 1000 grain weight. (Marri et al. 2005; Thalapati et al. 2012). On the other hand, five genotypes (S-166-2, S-166-30, K-137, K-363–12 and K-463) were identified as heat susceptible genotypes.

All four N22 mutants showed higher spikelet fertility in heat stress conditions compared to N22. The new genetic variations obtained from either spontaneous or induced mutants using physical or chemical mutagens can be used in crop breeding and functional genomics (Poli et al. 2013). Though heat stress was the main effect that was considered in both the seasons, it cannot be ruled out that part of the effect may have been contributed by slightly decreased radiance in the poly cover house during wet season and the effect of some environmental variables other than heat in the late sown conditions of dry season.

Both STRUCTURE analysis and the clustering using UPGMA showed similar results and the best possible groups in both analyses were five. However, there was slight variation in grouping genotypes, for example KMR3 ILs were grouped into two in clustering method where as in STRUCTURE analysis, KMR3 ILs were in one group. These minor changes are expected because clustering was based on empirical groupings by dissimilarity values and bootstrapping constancy where as

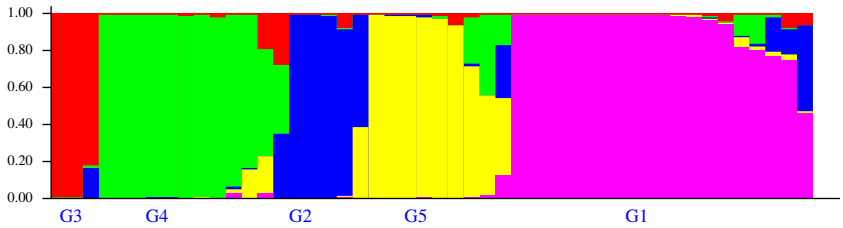

Fig. 5 Estimated population structure of 48 genotypes (K = 5). The y-axis shows group membership and x-axis is the 48 genotypes and G stands for Group

structure is model based, assuming the loci are at Hardy-Weinberg equilibrium and linkage equilibrium within populations. One of the Swarna ILs, S-148 may have more introgressions from *Oryza nivara* and thus share more alleles

Table 4 Association of marker alleles with phenotypic traits using GLM (Q) and MLM (Q + K) models in wet season 2012 and dry season 2013 under normal and polycover house/late sown conditions

			Normal Conditions			Polycover house/late sown conditions		
				GLM	MLM		GLM	MLM
Trait	Marker	Chromosome	R^2	-log $_{10}$ *P* value	-log $_{10}$ *P* value	R^2	-log $_{10}$ *P* value	-log $_{10}$ *P* value
Wet season 2012 (Polycover house method)								
Flowering time	RM250	5	3.69	1.852		NS		
	RM88	8	NS			13.45	2.009	
Spikelet fertility	RM229	11	NS			29.93	2.226	1.522
Yield per plant	RM570	3	15.65	1.670		NS		
	RM250	5	20.96	1.578		NS		
	RM235	12	23.18	1.518		NS		
Dry season 2013 (Late sown method)								
Flowering time	RM235	12	15.33	2.112	1.645	NS		
	RM3181	8	12.15	2.326		NS		
	RM282	3	18.86	2.320	1.584	NS		
	RM440	5	16.04	1.803		NS		
	RM547	8	13.77	1.592		NS		
	RM3735	4	NS			9.98	2.299	
	RM167	11	NS			8.75	1.970	1.563
	RM131	4	NS			5.28	1.590	
	RM128	1	NS			4.26	1.502	
Spikelet fertility	RM108	9	10.21	1.617		16.18	1.868	1.669
	RM167	11	13.81	2.241		11.77	2.366	1.675
	RM282	3	16.70	2.171		NS		
	RM131	4	25.33	2.165	1.916	NS		
	RM210	8	NS			13.20	2.323	
	RM430	5	NS			11.74	2.213	1.663
Yield per plant	RM430	5	NS			12.84	1.586	
		Wet season 2012				Dry season 2013		
HSI for spikelet fertility	RM229	11	38.17	2.647	1.762	NS		
	RM210	8	NS			12.79	2.076	
	RM430	5	NS			11.01	1.924	1.512
HSI for yield per plant	RM274	5	36.16	2.317		NS		
	RM545	3	NS			29.86	1.941	1.519
	RM430	5	NS			17.87	1.537	

NS not significant

with *Oryza nivara* or some novel variations may have been created in this IL. The latter is more likely as S-148 showed 48 null alleles at 79 SSR loci tested (unpublished) indicating widespread changes in genome which resulted in its not being with other Swarna ILs. Phenotypically also IL 148 is significantly taller, early flowering and has slender grains compared to Swarna.

In the present study, 8 marker trait associations in wet season 2012 and 20 marker trait associations in dry season 2013 were identified. During wet season, RM229 on chromosome 11 was significantly associated with both spikelet fertility and its HSI explaining 30 % phenotyping variance by both GLM and MLM in poly cover house method. RM229 allele which is also present in heat tolerant checks Bala and N22, at this locus contributed significantly to increased spikelet fertility under heat stress as per allele estimates output of GLM and MLM models. Cheng et al. (2012) also identified RM229 flanking spikelet fertility QTL *qSF11* in the advanced backcross population (BC_2F_6) of Xiushui 09/IR2061. In our previous study, RM229 was significantly linked to yield in normal conditions in the F_2 population of IR64 and NH219, a heat tolerant mutant of N22 (Poli et al. 2013). It was also linked to yield and panicle number in a BC_2F_2 population derived from inter specific cross between Caiapo, an upland *O. sativa* (japonica) variety from Brazil and *O. rufipogon* from Malaysia (Moncada et al. 2001). Thus RM229 appears to be important for both spikelet fertility under heat stress and yield under normal conditions.

Heat stress during flowering causes more reduction in yield due to low pollen fertility and low seed set. Several QTLs have been mapped for heat tolerance at flowering stage (Ye et al. 2011; Jagadish et al. 2010a; Zhang et al. 2008; Xiao et al. 2011). In our study, RM250 and RM88 were associated with days to flowering in normal and poly cover house methods respectively but same type of alleles were observed in both susceptible and tolerant checks indicating they are not specific to a haplotype. RM88 was present in the same position of RG978 marker, which flanks *qhr8–1* for heat tolerance in DH population of IR64 and Azucena (Cao et al. 2003; Temnykh et al. 2001). One QTL was identified at marker locus RG256 (at 33.96 Mb) on chromosome 2 and associated with spikelet fertility under control conditions (Jagadish et al. 2010a). This locus is very close to RM250 at 32.79 Mb. Out of three markers (RM235, RM570, RM250), linked to yield per plant in control conditions, allele of RM235, contributing to heat tolerance was observed only in heat tolerant checks. The alleles of the other two markers RM570 and RM250 were not specific to either heat tolerant or heat susceptible checks. RM235 was associated with spikelet fertility QTL *qHt12* on chromosome 12 with 10 % phenotypic variance in heat stress treatment during flowering stage (Chen et al. 2008). This marker was also linked to leaf width in RIL population of cross Lemont × Teqing and two backcross hybrid populations derived from these RILs (Mei et al. 2005). Jagadish et al. (2010a) reported *qtl_2.3* for spikelet fertility in control conditions and the allele was contributed by Azucena, a susceptible parent. RG256 marker was a flanking marker of this QTL and was 7 cM away from marker RM250 (Temnykh et al. 2001). Marri et al. 2005 also reported that RM250 was a marker interval of QTL for panicle length (*pl2.1*) in addition to spikelet number and grain weight in BC_2 testcross progeny of KMR3 introgression lines. Allele at locus RM274, associated with HSI of yield per plant was contributed by N22 and NH219, which were reported earlier as heat tolerant (Jagadish et al. 2010b; Panigrahy et al. 2011; Poli et al. 2013). This marker was also linked to spikelet fertility in both control and heat stress conditions in RIL population of Zhongyouzao8 x Toyonishiki and the allele for heat tolerance was from the heat tolerant parent, Zhongyouzao8 (Zhang et al. 2008).

During dry season 2013, five markers (RM235, 3181, 282, 440 and 547) were associated with days to flowering in normal sowing and four markers (RM3735, 167, 131 and 128) were associated with days to flowering in late sown condition. Allele types at locus RM167 in N22 and RM235 in NH219 contributed to this trait. In case of other loci, the contributing allele types were found in both susceptible as well as tolerant checks and thus cannot be considered specific to heat tolerance. RM282 was also reported earlier as flanking marker for days to heading QTL, *qdth3.2* in rice and the positive allele was from the cultivar Jefferson. Allele from *O. rufipogon* at this locus increased the shattering effect (Thomson et al. 2003). In our study, RM108 on chromosome 9 and RM167 on chromosome 11 were significantly related to spikelet fertility in both normal and heat stress conditions. RM108 was associated with spikelet fertility QTL, *qHt9a* in RIL population of T219 x T226 cross under heat stress treatment (Chen et al. 2008). RM108 was also associated with 1000 grain weight and panicle length in double haploid population of cross WYJ2(J) × Zhenshan 97(I) (Jiang et al. 2004). The estimated alleles for heat tolerance in case of RM167 were observed in N22, the heat tolerant check where as that of RM108 were in both tolerant and susceptible checks. In addition, two other markers RM210 and RM430 were also associated with spikelet fertility in heat stress conditions and the contributing alleles were similar to the alleles of tolerant checks Bala, N22 and NH219. Bui et al. (2014) also detected RM167 using Single Marker Analysis to be associated with filled grains per panicle and explaining 13.1 % of the total phenotypic variance under heat stress conditions. Thomson et al. (2003) and Xiao et al. (1998) reported that *O. rufipogon* allele of RM210 locus was a peak marker for yield QTLs *gpp8.2* and *gpl 8.1* and increased grain number in advanced backcross population between the U.S. cultivar Jefferson (*O. sativa* ssp. Japonica) and an accession of *O. rufipogon* (IRGC 105491). Luo et al. (2011) also showed that RM210 was a

heterotic locus, *hsp8* associated with number of spikelets per panicle in ILs of common wild rice *O. rufopogon* in an indica cultivar Guichao2 background. In our study, RM430 was significantly associated with both spikelet fertility and yield per plant explaining 11.74 % and 12.84 % phenotypic variance respectively under heat stress conditions. This marker was also linked to their relative heat tolerance i.e., heat susceptibility indices. The major contributing allele for heat tolerance at this locus was that of Bala and N22, both heat tolerant checks with similar allele. However, Cheng et al. (2012) found a QTL *qSF5* on chromosome 5 with maker interval of RM430-RM440, associated with spikelet fertility in normal conditions but not in high temperature conditions. RM430 was an interval marker for a relative grain yield QTL, *QRgw5*, identified in RIL population of a cross between an indica lowland cultivar Zhenshan97 and a tropical japonica upland cultivar IRAT109 under drought (Yue et al. 2006). Bai et al. (2011) also reported this marker as a flanking marker for a heading date QTL, *qhd5* in RIL population of a cross between diverse cultivars, Nanyangzhan and Chuan7.

Though RM545 was significantly associated with HSI for yield per plant but the estimated alleles were inconclusive as this allele was observed in 26 lines including both tolerant and susceptible lines out of 48 lines tested. This locus was close to RG100 which flanks QTL, *qhr3–1* for heat tolerance in DH population of IR64 and Azucena (Cao et al. 2003; Temnykh et al. 2001). Jagadish et al. (2010a) reported two QTLs for spikelet fertility under normal conditions on chromosomes 2 and 4 and eight QTLs for spikelet fertility under heat stress conditions on chromosomes 1, 2, 3, 8, 10, and 11 in recombinant inbred lines of Bala (tolerant) x Azucena (susceptible). There was no correlation between spikelet fertility in control and high-temperature conditions and no common QTLs were identified indicating different genomic regions are involved in governing spikelet fertility during heat stress. In our study also there were no common markers associated with spikelet fertility under both control and heat stress conditions in wet season 2012 experiment but two markers RM108 and RM167 were associated with spikelet fertility under both control and late sown (heat stress) conditions in dry season 2013.

Nagina22 is a drought and heat tolerant aus variety and IR64 and Azucena are considered heat susceptible (Jagadish et al. 2010b). We also show that allele type of N22 and Bala, the heat tolerant checks, contributed maximum number of loci reported to be linked to spikelet fertility and yield under heat stress conditions. Marri et al. (2005) reported RM263 and RM183 on chromosome 2 linked to yield enhancing QTLs *qyld 2.1* and *qyld 2.2* and RM297 and RM223 to high spikelet number per panicle. Xiao et al. (2011) reported two QTLs *qPF4* and *qPF6* between RM5687 and RM471 on chromosome 4 and between RM190 and RM225 on chromosome 6 respectively, affecting pollen fertility in RILs derived from a cross between a heat tolerant rice cultivar 996 and a sensitive cultivar 4628. However these markers were not associated with any trait in our study. Thus these markers associated with heat tolerance traits such as spikelet fertility may be cross/genotype -specific and not universally associated with the trait.

Molecular marker based mapping together with availability of the whole genome sequences and fast progress in functional genomics can help in the identification of candidate genes for heat tolerance. In the present study, 32 candidate genes were identified close to the 9 markers significantly associated with spikelet fertility and yield per plant in heat stress conditions. These genes are reported to be putatively functional under heat stress (http://rapdb.dna.affrc.go.jp/). These candidate genes are being used for expression profiling of heat tolerant and heat susceptible genotypes under heat stress.

Spikelet fertility, yield per plant and their HSI were considered important to assess heat tolerance in 48 rice genotypes in two seasons, wet season 2012 using poly cover house method and dry season 2013 using late sown method. Four KMR3 ILs (K-13-5, K-16-3, K-377-24, and K-458) and eight Swarna ILs (S-10-3, S-14-3, S-24, S-65, S-70, S-230, S-248 and S-250) were identified as heat tolerant genotypes based on significant differences between means in control *vs.* heat stress treatments and also over their control parents for spikelet fertility and yield per plant and their HSIs in both wet and dry seasons. The number of genotypes used is not large enough for a new study on association mapping where QTLs have to be mapped. However, the number is adequate (considering also our limitations of handling large population) for validating QTLs already reported as in our study and also several other previous such studies on wheat (44 genotypes, Tommasini et al. 2007) soybean (44 genotypes, Singh et al. 2008) and rice (40 genotypes, Vannirajan et al. 2012). Moreover, clear population structure was observed using 48 genotypes (including four sets of elite × wild derived introgression lines, landraces and mutants) thus permitting genome-wide association mapping. Even though the number of genotypes was less, the results confirmed several QTLs.

Under heat stress conditions, RM229 on chromosome 11 in wet season 2012 and RM430 on chromosome 5 and RM210 on chromosome 8 in dry season 2013 were significantly associated with both spikelet fertility and its HSI. The estimated alleles at these 3 loci, contributing for heat tolerance were similar to that of heat tolerant checks, Bala and N22. RM430 was significantly associated with HSI of both yield per plant and spikelet fertility. Three markers (RM250, RM131 and RM282) were significantly associated with both spikelet fertility and days to flowering. Only two markers RM108 and RM167 were associated with spikelet fertility under both normal and late sown (heat stress) conditions. Ten markers which were associated with spikelet fertility and yield per plant (one marker each under control and heat stress conditions in wet season 2012 and five markers under

control and three markers in dry season 2013) were associated with flowering time, the third trait that we considered in this study. Thirty two candidate genes were identified close to these markers for further studies. Marker trait associations and heat tolerant lines identified in the present study will be useful in breeding heat tolerant lines for sustaining rice production under warmer climates. The purpose of the study was to validate QTL/markers already reported for heat tolerance which can then be assessed for their utility in MAS. If the shortlisted markers/candidate genes are closely linked to heat tolerance traits in a segregating mapping population and have a major effect, functional markers can be designed for use in MAS. As a part of climate change project NICRA in our institute, screening of large number of germplasm lines and further validation of these markers is under progress. A scheme of introgression of linked QTL alleles and markers from heat tolerant lines can be suggested as in any other QTL introgression schemes provided the markers are closely linked and known to have major effect on the trait. This should be demonstrated in different genetic backgrounds as QTL effect is context specific.

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