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ORIGINAL ARTICLE

A trait specific QTL survey identifies NL44, a NERICA cultivar as a novel source for reproductive stage heat stress tolerance in rice

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Abstract Rice is sensitive to heat stress at gametogenesis and anthesis stages. For sustaining rice yields under the predicted threat of reproductive stage heat stress (RSHS), identification of tolerant donors as well as mapping of genes governing tolerance is crucial. Recently a NERICA (NEwRIce for AfriCA) rice genotype, NL44 has been reported tolerant to RSHS. The present study aims to survey a recombinant inbred line (RIL) population developed from the cross, Pusa Basmati 1 (PB1)/NL44 using markers linked to 54 RSHS quantitative trait loci (QTLs) through phenotypic and genotypic characterization. When exposed to RSHS, the susceptible parent PB1 and several RILs showed significant reduction for spikelet fertility and grain yield plant^{-1} relative to NL44. Both these traits and the estimated stress tolerance index (STI) showed a quantitative pattern of inheritance. Out of the 116 SSR markers surveyed, 31 markers were polymorphic between PB1 and NL44. No discernible associations could be found through a preliminary bulked segregant analysis with these markers. A subsequent single marker analysis revealed five minor QTLs, four for spikelet fertility under heat stress and

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two for STI-spikelet fertility, of which one QTL was mapped for both the traits. These QTLs, however, could explain a very low level of total phenotypic variation. Additionally, the cumulative additive effect of these QTLs could account only for a possible 30% of the contrast between PB1 and NL44. Thus, the study clearly establishes that NL44 has novel genomic regions for RSHS tolerance.

Keywords Heat stress · Grain yield plant^{-1} · SSR · Recombinant inbred line (RIL) · Spikelet fertility

Introduction

Climate change poses a serious threat to agricultural productivity worldwide. Since early twentieth century, the earth's mean surface temperature has risen by 0.8 °C, of which about 0.6 °C increase is encountered since 1980 (IPCC 2007). Predictions for future imply that, relative to 1986–2005 period, global mean surface temperature is likely to increase by 0.3–4.8 °C by 2081–2100 period under different greenhouse gas emission scenarios (IPCC 2014). Due to climate change, impending heat waves beyond 35 °C have been predicted in South Asia, particularly in India, at the densely populated agricultural hotspots of the Ganges and Indus river basins (Im et al. 2017). Since rice is sensitive to heat stress, it is shown to result in significant reduction in grain yield under such adverse occurrences (Jagadish et al. 2007, 2010). Tolerance threshold of rice to heat stress is reported to be around 33 °C (Yoshida et al. 1981; Jagadish et al. 2010). Estimates with various simulation models suggest that rice will record yield loss ranging between 2.5 and 10.0% for every 1 °C rise in earth's surface temperature (Baker et al. 1992; Peng et al. 2004). Being a tropical country, the adversities due to heat stress is expected to be more pronounced in India, than in other rice growing Asian countries (Zhao et al. 2016). Episodes of rice yield decline due to heat stress have already been documented in several countries including India, Pakistan, Bangladesh, China, Thailand, Japan, Australia and the U.S.A. (Osada et al. 1973; Matsushima et al. 1982; Yang et al. 2004; Hasegawa et al. 2009; Tian et al. 2009; Welch et al. 2010).

Response of rice to heat stress differs with the growth stages. Heat stress sensitivity is the highest during early reproductive stages coinciding with booting followed by the anthesis (Satake and Yoshida 1978; Yoshida et al. 1981). Susceptibility at these stages results in severe yield penalty because anthers and pollens suffer severe desiccation leading to poor fertilisation and ultimate yield loss, particularly at prolonged temperatures over 35 °C. Additionally, the injury to male reproductive organ is manifested as abnormal anther dehiscence, poor pollen production, and low pollen germination rate (Osada et al. 1973; Matsushima et al. 1982; Matsui et al. 1997, b; Jagadish et al. 2007, 2008, 2010; Prasad et al. 2006).

Rice exhibits diverse adaptation to heat stress particularly rendering the classification as upland and lowland cultivars. Upland cultivars show better tolerance to heat stress especially during terminal stages than wetland cultivars. Heat stress tolerance in rice has been reported to be governed by a number of quantitative trait loci (QTLs) (Jagadish et al. 2010; Ye et al. 2012, 2015a, b; Shanmugavadivel et al. 2017; Li et al. 2018). QTLs for reproductive stage heat stress (RSHS) tolerance in rice have been reported on all the chromosomes (Arshad et al. 2017). Among the traits related to RSHS, spikelet fertility has been the most frequently analysed trait. Besides, floral traits such as anther length and anther dehiscence have also been targeted (Tazib et al. 2015). In order to map these QTLs, markers such as simple sequence repeats (SSRs), restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP) have been utilized in several studies (Ye et al. 2012, 2015a, b; Shanmugavadivel et al. 2017). However, most of the QTLs reported, except those by Ye et al. (2015a), have not been fine mapped and validated. Among the donors for RSHS tolerance, Nagina 22 (N22) has been the most widely studied.

Development of resilient cultivars tolerant to heat stress is an important pre-emptive strategy for sustaining rice production under the projected rise in temperature. Considering the complexity of RSHS tolerance and the growing importance of this problem, it is essential that diverse donors be identified, and QTLs mapped. An attempt towards this has resulted in the identification of a heat tolerant rice genotype among the NERICA (NEwRIce for AfriCA) cultivars, Nerica L 44 (NL44). A comparative study of this line *vis-a-vis* N22, an established RSHS tolerant cultivar, has identified NL44 to perform at par with N22 in terms of spikelet fertility and physiological resilience, making it a strong candidate for further studies (Bahuguna et al. 2015). However, the genomic regions imparting the high RSHS tolerance of NL44 has not yet been identified. To address this, a recombinant inbred line (RIL) population was developed at the Division of Genetics, ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi involving Pusa Basmati 1 as the RSHS sensitive parent and NL44 as the tolerant parent. Keeping in perspective of the above, as a first step enroute to QTL mapping, the present investigation was carried out to check for the presence of any previously reported RSHS tolerance QTLs in NL44, by investigating marker trait association in the RIL population.

Materials and methods

Plant material

A population of 127 RILs was developed through single seed descent method from the cross Pusa Basmati 1/NL44. The RSHS sensitive parent, Pusa Basmati 1 is the world's first high yielding semi-dwarf Basmati rice variety released by ICAR-IARI, New Delhi. Developed through convergent breeding strategy from the cross Pusa150/Karnal local (Siddiq et al. 2009), Pusa Basmati 1 has become highly popular among the farmers and the consumers, particularly for its extra-long grain type, grain and cooking quality and mild aroma. Albeit it's superior qualities, this variety is highly sensitive to various biotic and abiotic stress in general and RSHS in particular. As mentioned previously, NL44 shows significant RSHS tolerance exhibiting higher seedling survival rate, superior growth and greater reproductive resilience, even when exposed to 42.2 °C under field conditions (Bahuguna et al. 2015). Furthermore, NL44 maintains higher spikelet and pollen fertility, as that of the widely documented RSHS tolerant *aus* variety N22 (Yoshida et al. 1981; Prasad et al. 2006; Jagadish et al. 2008; Rang et al. 2011). The RILs were advanced to F_7 generation, following standard agronomic management.

Heat stress screening

The RILs along with the parents were evaluated under natural field conditions at IARI – Rice Breeding and Genetics Research Centre at Aduthurai in Tamil Nadu (11.0154° N, 79.4809° E). Two staggered sowings were made during the *Rabi* season of 2017–2018, with the first sowing timed to coincide the normal growing season and the second sowing to coincide with the heat stress during flowering. This ensured two contrasting environments, the first sown set did not experience any stress (unstressed) while the second set experienced the heat stress (stressed) beginning from anthesis. Experiments were laid out in an incomplete block design, with parents replicated seven times. Plots were of uniform size with 3 m length and 0.6 m width. A spacing of 20 cm between rows and 15 cm between plants was maintained. At flowering, five randomly selected plants per genotype were tagged and pollen fertility (%) was determined using I_2-KI staining method. For this, the primary tiller florets that were exposed to heat stress were sampled. The day prior to the anthesis, three spikelets per each tagged plant were fixed in Carnoy's fixative (three parts of 70% ethanol mixed with one part of glacial acetic acid). Three anthers from each spikelet were processed in I_2-KI stain. The stained pollens were observed under a Nikon Alphaphot-2 YS2 compound microscope (Tokyo, Japan) using 100 × magnification. The pollen grains which imbibed stain and appeared dark blue were considered fertile, while the unstained ones were treated sterile. The proportion of fertile to total number of pollen grains per microscopic field was used to calculate pollen fertility and expressed in percentage. After physiological maturity, the tagged plants were also used to measure traits such as grain yield plant^{-1} and spikelet fertility (%). For estimating spikelet fertility, the panicle from primary tiller was sampled. Spikelet fertility was calculated as the ratio of filled spikelets to total number of spikelets per panicle and expressed as percentage. The observations averaged over five plants were used for statistical analyses. Further, stress tolerant index (STI; Fernandez 1992) was calculated for grain yield plant^{-1} and spikelet fertility to assess the relative performance of RILs under heat stress using the following formula,

$$\frac{(Ys)(Yp)}{Yp^2}.$$

where Ys and Yp are the average yield/spikelet fertility of genotypes under stressed and unstressed conditions, respectively, while Yp represents mean yield/spikelet fertility of all genotypes under unstressed condition.

Molecular marker selection and bulked segregant analysis

We have used two approaches to narrow down to the putative QTLs possessed by the NL44 for RSHS tolerance. For this, an exhaustive survey of literature was conducted to inventory the RSHS QTLs reported in rice. Information on the linked SSR, SNP and RFLP markers were collated. Around 116 SSR markers were selected for polymorphism survey between the parents (NL44 and Pusa Basmati 1). N22 was used as a positive control. For molecular analysis, the total genomic DNA was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) method from young leaves (Doyle and Doyle 1990). The DNA concentration and quality were checked using a Nano spectrophotometer (Microdigital Co. Ltd., Korea). For PCR amplification, a total of 10 µl reaction mix was assembled with 50 ng of template DNA, 5 pmol each of both forward and reverse primers, 0.2 mM dNTPs, 1.5 mM of $MgCl_2$ and 0.5 U of Taq DNA polymerase (Genei Laboratories Pvt. Ltd., Bengaluru). The PCR program consisted of an initial denaturation for 5 min at 95 °C, followed by 35 cycles consisting of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 1 min extension at 72 °C. After 35 cycles, the final extension was rendered at 72 °C for 7 min and the products were cooled to 4 °C. The amplicons were resolved through electrophoresis in 3.5% agarose gel (Seakem LE Agarose, Lonza, USA) mixed with 0.1 mg/ml

of ethidium bromide. A 100 bp ladder DNA (GeneDireX, USA) was used as standard for comparison between amplicons by electrophoresis and visualisation under trans-illumination in a gel documentation system (BioRad, USA).

In the initial approach to run a bulked segregant analysis (BSA), only those markers which were polymorphic between the parents were utilized. Thirteen genotypes (10% of RILs, i.e., $\sim$ 13) that were found to show extreme phenotypes for grain yield plant^{-1}, spikelet fertility and pollen fertility were selected to construct two DNA pools, one for tolerant and the other for sensitive. The genomic DNA of the selected RILs was normalized to a concentration of 50 ng µl^{-1} using TE buffer and equal volume of DNA (10 µl) was pooled to construct the bulks. The polymorphic SSR markers were then surveyed over the bulks and the parents. In the second approach, all the polymorphic markers were analysed over the entire RIL population. A simple linear regression was conducted to test the linkage between the putative markers and the trait variables, in order to determine the phenotypic variance explained.

Results

The two sowing windows used in the experiment provided adequate exposure to different temperatures during the flowering period (Fig. 1). In the first sowing window, the maximum temperature ranged from 32 to 37 °C during active flowering and grain formation, with an average of 35.5 °C. While in the second window, the maximum ambient temperature exceeded the critical minimum temperature of 35 °C completely during the flowering period. The maximum temperature during this period ranged between 37.0 and 42.0 °C with an average temperature of 40.3 °C.

Phenotypic characterization of the RILs and parents

The analysis of variance (ANOVA) revealed significant difference between the two sowing windows for all the traits (Table 1), while the genotypes including the parents and the RILs showed insignificant differences barring grain yield. The genotype by sowing time interaction was also significant for spikelet fertility and grain yield. Among the parents, under normal planting condition when plants were unstressed, average grain yield plant^{-1} of NL44 was 22.7 g and that of PB1 was 23.2 g (Table 2). Corresponding spikelet fertility was 77.8% and 71.0% in NL44 and PB1 respectively. However, the pollen fertility did not vary among the parents under unstressed conditions. Whereas, under late planted situation, when the plants experienced RSHS, the sensitive parent PB1 recorded a grain yield of 7.5 g plant^{-1} as against 14.6 g plant^{-1} in NL44 (Fig. 2). The yield reduction in NL44 was 35.7%, while in PB1 it was 67.7%. Similarly, significant reduction of pollen and spikelet fertility were also observed in PB1, while in NL44 the spikelet fertility ($\sim$ 77.0%) and pollen fertility ($>$ 93.0%) remained almost similar in both stressed and unstressed conditions. Relative reduction is spikelet

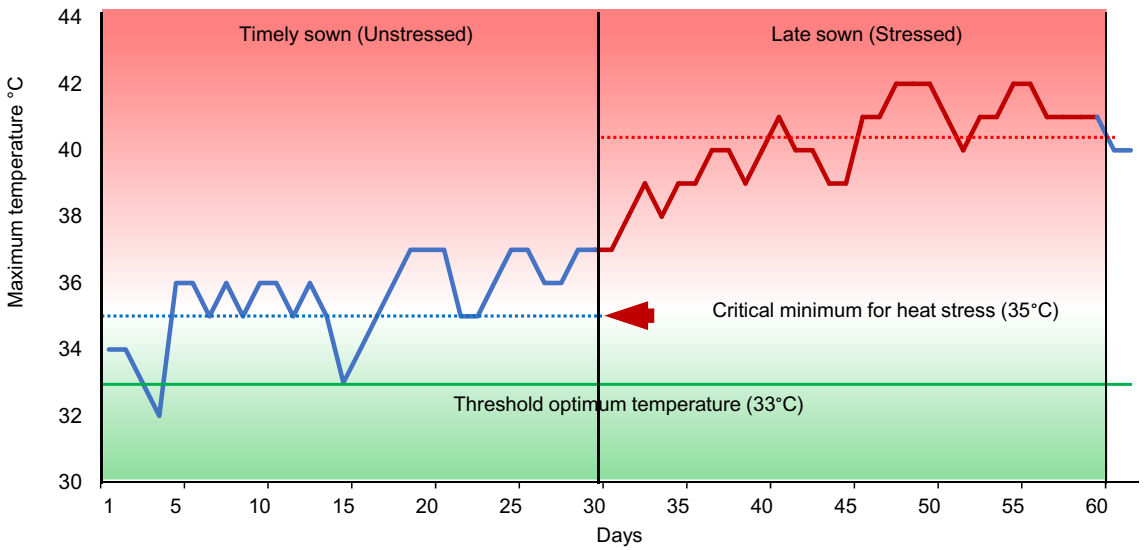

Fig. 1 Daily maximum temperatures experienced during the flowering period by the RILs sown timely and late, indicating the exposure to high temperature. The dotted lines indicate the average reproductive stage maximum temperatures for the two sowing windows. *The 30-day window indicated for timely sown (unstressed) and late sown (stressed) set of RILs coincides with 80–110 days after sowing

Table 1 Analysis of variance (ANOVA) of different traits between two staggered sowing

Source of variation	*Df*	Mean squares		
		Pollen fertility (%)	Spikelet fertility (%)	Grain yield plant^{-1} (g)
Sowing time	1	3217.5**	26,539.4**	5689.3**
Genotypes	256	49.7	120.0	3997.5**
Genotypes × sowing time	256	48.6	97.8**	1482.9**

Df degrees of freedom

**Significant at 99% confidence level

Table 2 Summary statistics for grain yield. plant^{-1}, spikelet fertility (%) and pollen fertility (%) recorded in the 127 RILs and parents under timely sown (unstressed) and late sown (high temperature stressed) conditions

Statistic	Grain yield. plant^{-1} (g)		Spikelet fertility (%)		Pollen fertility (%)	
	Unstressed	Stressed	Unstressed	Stressed	Unstressed	Stressed
Range	9.6–38.2	2.1–22.7	64.0–90.8	16.6–82.2	97.0–100.0	40.4–99.9
Mean	22.4	13.0	79.1	58.9	98.2	90.7
SE	0.45	0.37	0.51	1.17	0.06	0.88
SD	5.08	4.17	5.74	13.23	0.74	9.89
Kurtosis	0.09	− 0.45	− 0.29	− 0.16	− 1.01	8.50
Skewness	− 0.15	0.09	− 0.36	− 0.52	− 0.38	− 2.74
NL44	22.7	14.6	77.8	77.2	100.0	93.3
PB1	23.2	7.5	71.0	24.8	99.0	86.3
CV (%)	22.7	32.0	7.3	22.5	0.73	10.9

SE standard error, *SD* standard deviation, *CV (%)* coefficient of variation in per cent

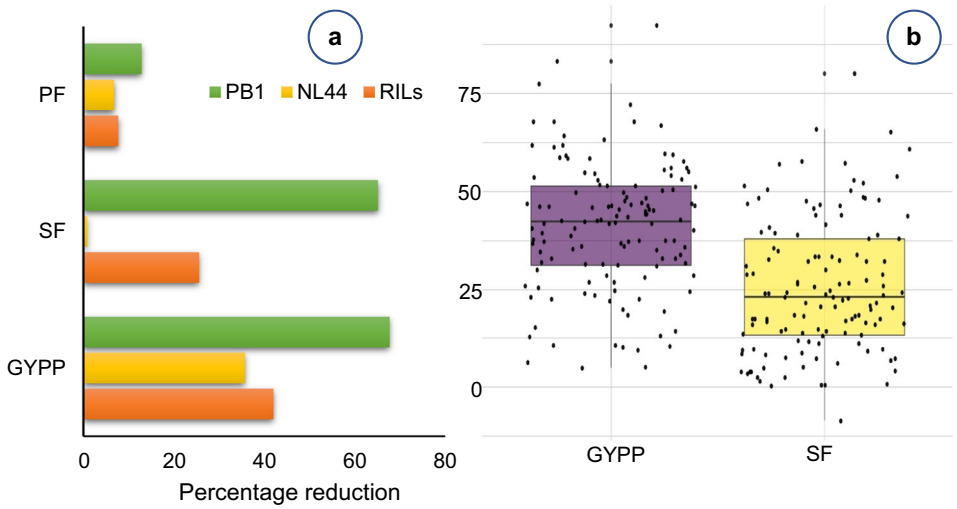

Fig. 2 Relative reduction in different traits **a** expressed in percentage among parents and RILs under reproductive stage temperature stress. Traits are pollen fertility (PF), spikelet fertility (SF) and grain yield plant^{-1} (GYPP). **b** Box plots with jitters depicts the distribution of per cent reduction values of genotypes for GYPP and SF

fertility in PB1 was 65.1%, with a corresponding 12.8% reduction in pollen fertility.

Among the RILs, however, the mean grain yield plant^{-1} ranged between 9.6 and 38.2 g plant^{-1} with a mean of 22.4 g under unstressed conditions (Table 2). While under RSHS, the per plant grain yield among the RILs ranged from 2.1 to 22.7 g, with a mean of 13.0 g. With spikelet fertility, the RILs showed a range of 64.0–90.8% under unstressed conditions, and 16.6–82.2% under stress. Corresponding averages in spikelet fertility among the RILs were 79.1% and 58.9%, respectively for stressed and unstressed treatments. Drawing a parallel to the parental performance, pollen fertility among the RILs was in a range of 97.0–100.0 with a mean of 98.2% under unstressed situation. Similar reduction was also observed in the RILs in comparison to the control. The per cent reduction under stress over non-stress showed that the grain yield reduction was relatively higher (mean reduction of 41.9%)

compared to per cent spikelet fertility (mean reduction of 25%) (Fig. 2). Furthermore, stress tolerance index (STI) was calculated for grain yield plant^{-1} and spikelet fertility as it is more indicative of relative tolerance of RILs (Table 3). The contrast between parents was more vivid in terms of STI calculated for spikelet fertility than for the grain yield *per se*.

The RILs exhibited normal distribution for grain yield and spikelet fertility. Whereas, pollen fertility showed a skewed distribution towards fertility (Fig. 3). The frequency distributions for these traits are indicative of their quantitative nature of inheritance. Grain yield plant^{-1} showed a near normal distribution followed by spikelet fertility under both stressed and unstressed conditions. Also, there were RILs with extreme values than the parents both under stressed and unstressed conditions, indicating transgressive segregation of heat stress response. The STI calculated from grain yield and spikelet fertility also showed near normal distributions.

Significant correlation was observed between grain yield plant^{-1} and spikelet fertility under heat stress conditions (Fig. 4). Pollen fertility showed very weak correlation with either grain yield per plant^{-1}, spikelet fertility and also with the STI computed from these traits. This is further evident from significant regression coefficient of spikelet fertility over grain yield (Supplementary Fig. 1). Despite the exposure to high temperature stress, pollen fertility among the RILs as well as the parents was relatively less affected. Contrary to the expectations, at higher temperature majority of the genotypes recorded higher pollen fertility, although their spikelet fertility remained lower (Supplementary Fig. 2). This loss of apparent correlation between pollen and spikelet fertilities could also be observed as high skewness of pollen fertility (− 2.74).

Quantitative trait loci (QTLs) for reproductive stage heat stress tolerance

Already reported 109 QTLs associated with RSHS tolerance and related traits are compiled in Fig. 5. A detailed list of these QTLs is summarized in Supplementary Table 1. Apparently, all the chromosomes are reported to hold QTLs associated to RSHS response. The number of QTLs reported was highest on chromosome 4 (18 QTLs) followed by chromosome 1 (15 QTLs) and chromosome 3 (13 QTLs). Remaining chromosomes has number of QTLs ranging from two (Chromosome 12) to eleven (chromosome 5). Distinct 'QTL hot spots' could be delineated on chromosome 1 within the genomic region spanning from 38 to 40 Mb. There was a QTL hotspot found on chromosome 2 between 20 and 25 Mb region, that harboured five QTLs. On chromosome 4, there were two hotspots, one within 26–33 Mb region having eight QTLs, and another between 15 and 20 Mb with seven QTLs. Hotspot on chromosome 5 was within a shorter span between 0.1 and 3.1 Mb region, but possessed seven reported QTLs. On chromosome 8, four QTLs were located within a 1 Mb span, beginning from 24 Mb position. Further, on chromosome 10, there were four QTLs located within 18.7–20.8 Mb region.

Majority of the earlier mapping studies for RSHS tolerance used SSR markers, while a few recent studies used SNP markers (Ye et al. 2012, 2015a, b; Shanmugavadivel et al. 2017). Also, there are few initial studies that used RFLP markers. Among the donors investigated, most common donors were 966 and N22, followed by Azucena, Habataki and Kasalath. Different types of mapping populations such as F_2, RILs, backcross inbred lines (BILs), doubled haploids (DH) and chromosome segment substitution lines (CSSLs) were used in different studies. The size of these populations ranged from 37 CSSLs (Zhao et al. 2016) to 310 BC_2F_2 lines (Buu et al. 2014). Many of the studies were conducted under controlled glass house conditions and the temperatures were maintained above 35 °C for heat stress treatment, while ~ 31 °C was used as unstressed treatment. However, in some of the studies, genotypes were exposed to heat stress under natural field conditions by adjusting their sowing dates to coincide the stress onset as done in the present study (Buu et al. 2014; Tazib et al. 2015; Zhao et al. 2016; Zhu et al. 2017; Seo et al. 2019). Another important feature of the earlier studies was the use of spikelet fertility as the single most important trait for mapping RSHS tolerance. Interestingly, grain yield was the least explored trait. Additionally, there was also a wide variation in the proportion of phenotypic variation explained (PVE) for the target trait reported with the QTLs. Reported PVEs ranged from 6 to 36% (Buu et al. 2014). Based on these studies, we have compiled a set of

Table 3 Summary of heat stress indices measured for grain yield. plant^{-1} and spikelet fertility among the RILs from PB1/NL44 cross

Indices	RILs					NL44	PB1
	Minimum	Maximum	Mean	Range	SE		
$STI_{(sf)}$	0.22	1.12	0.74	0.90	0.01	0.96	0.28
$STI_{(gy)}$	0.10	1.57	0.59	1.46	0.02	0.66	0.35

$STI_{(sf)}$, stress tolerance index for spikelet fertility; $STI_{(gy)}$, stress tolerance index for grain yield. plant^{-1}; SE, standard error

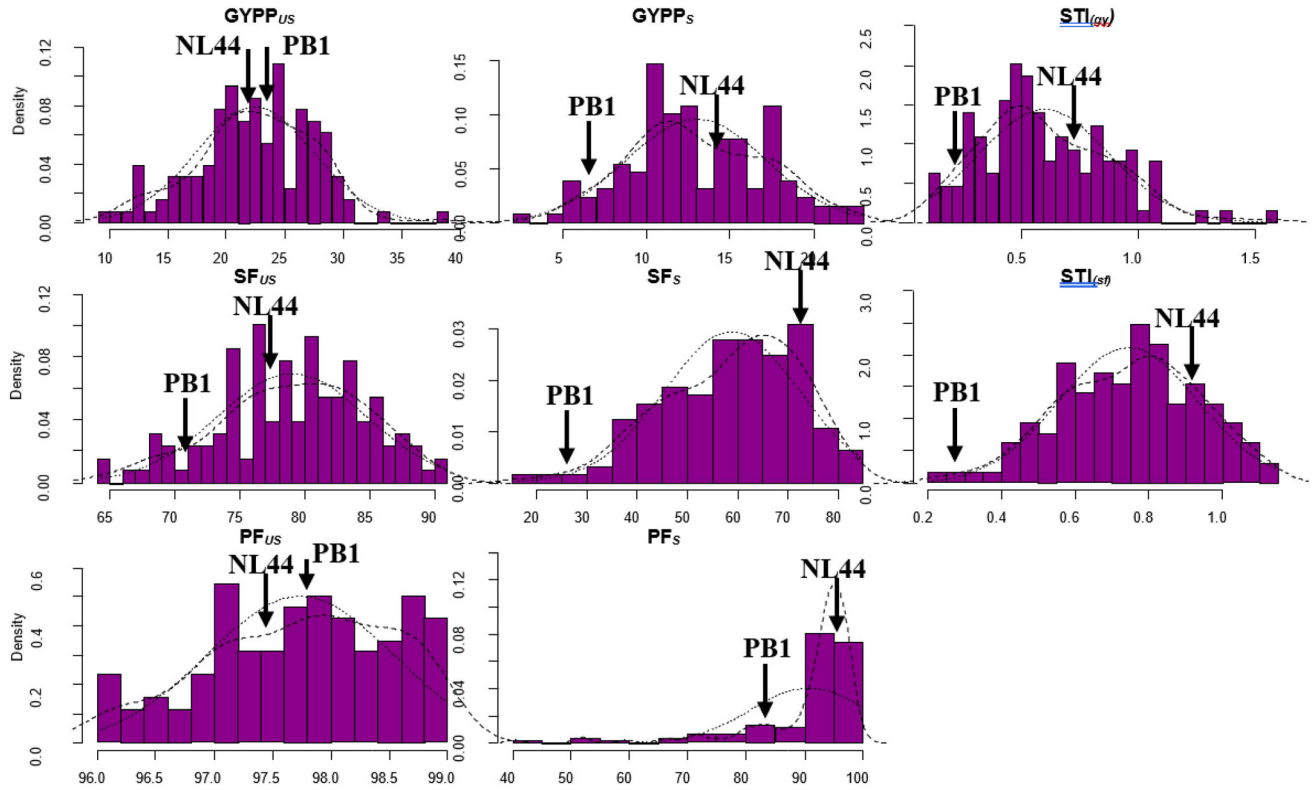

Fig. 3 Frequency distributions of grain yield plant^{-1} (GYPP), spikelet fertility % (SF) and pollen fertility % (PF) under unstressed (*US*) and stressed (*S*) conditions with reproductive stage heat stress show near normal distribution. The stress tolerance indices calculated for grain yield per plant STI$_{(gy)}$ and spikelet fertility % STI$_{(sf)}$ also show normality of distributions

116 QTL linked SSR markers for further analysis in the present study (Supplementary Fig. 3).

Parental polymorphism

Out of a total of 109 QTLs investigated, 54 QTLs were shortlisted based on the use of SSR markers and 116 linked SSR markers have been used that were distributed across all chromosomes (Supplementary Fig. 3). Genome wide survey of the two contrasting parents for RSHS response, PB1 (sensitive) and NL44 (tolerant) using the selected SSR markers had resulted in identification of 31 polymorphic markers (Table 4), indicating a putative 26.7% diversity between them. Of the polymorphic markers, the highest number was found on chromosome 4 (7 markers) followed by chromosome 3 (5 markers) whilst the least number of markers were from chromosome 7, 9 and 12 (1 marker).

Bulked segregant analysis

Thirteen genotypes that represent about 10% of the RILs, were drawn from extremes of phenotypic distribution based on grain yield plant^{-1} (Supplementary Fig. 4). The lines were also compared for the effect on spikelet fertility. The criteria for selection was the highest and lowest per cent of reduction for the target trait under heat stress over control. The lines with higher reduction were used to constitute the sensitive bulks, while those with lowest percent of reduction was used for constituting the tolerant bulk. The marker-based survey of the bulks along with the parents, however, could not resolve the phenotypic difference between the bulks.

Single marker analysis

Since no putative linkage between the marker and the traits could be established through BSA, we could assume that none of the already reported markers are adequate enough to associate to the RSHS tolerance in NL44. However, to resolve any weaker association, all the 31 polymorphic markers were run on the entire RIL population. Based on the segregation pattern, a subset of 19 markers that showed stark differences between the parents were scored. The subsequent single markers analysis revealed few significant associations (Table 5), particularly with spikelet fertility. The PVE, however, ranged between 3.3 and 6.9. The highest PVE was obtained for RM6394 for STI calculated for spikelet fertility (STI$_{sf}$), while the lowest was with RM7283 for spikelet fertility under stress (SF$_S$). Only one marker RM401 showed significant association both for

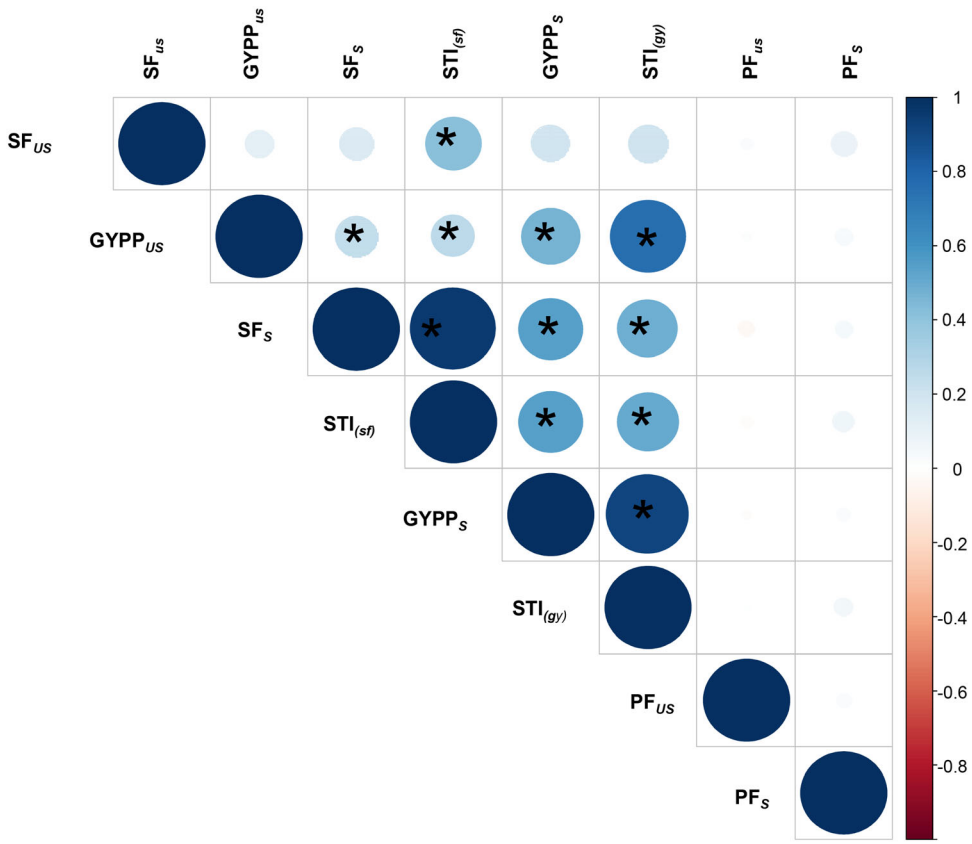

Fig. 4 Correlation plot between grain yield plant^{-1} (GYPP), spikelet fertility % (SF) and pollen fertility % (PF) under unstressed (US) and stressed (S) conditions with reproductive stage heat stress as well as with the estimated stress tolerance indices for grain yield plant^{-1} $STI_{(gy)}$ and spikelet fertility % $STI_{(sf)}$. The strength of the association is depicted by the colour code given in the legend and the significant (p value < 0.05) associations were indicated by asterisk

spikelet fertility under heat stress (SF_S) and STI for spikelet fertility (STI_{sf}). No significant associations were obtained for grain yield plant^{-1} under stress and its respective STI. This warrants a detailed QTL mapping study involving genome wide markers to identify the genomics regions governing reproductive stage heat tolerance in NL44 using the RIL population.

Discussion

As per the IPCC fifth assessment report, heat stress coinciding with the reproductive stages of various crops is a serious threat to global food security (IPCC 2014). Frequent occurrence of heat stress could deter the crop yield potential in major food crops in general and rice in particular for two obvious reasons (1) it is the most important crop that is grown exclusively under flooded environments, and extensively in the most populated parts of the world (Redfern et al. 2012), and (2) is highly sensitive to heat stress at reproductive stages (Satake and Yoshida 1978; Yoshida et al. 1981). Although estimates of yield loss due to RSHS in rice around the world indicates an incoherent pattern, yield reduction up to 60% or higher can be expected depending upon the rice ecology and the duration and intensity of high temperature exposure. Rice breeding programmes are to be devised to develop varieties tolerant to heat stress in order to be better equipped for the predicted temperature spikes.

Reduction of spikelet fertility and the consequent grain yield are the important ramifications of heat stress encountered in rice. In agreement to this fact, spikelet fertility and grain yield plant^{-1} were significantly affected under heat stress at reproductive stage in the present study, particularly among the sensitive parent PB1 and sensitive set of RILs. This was ascertained by the clear contrast observed between parents, NL44 and PB1. The estimated advantage of the tolerant parent, NL44 over PB1 was two times in terms of yield, and three times in terms of spikelet fertility. This establishes the superiority of NL44 as a RSHS tolerant cultivar, a candidate donor for future rice improvement programmes. Transgressive segregants for spikelet fertility, particularly exceeding tolerant parent in the mapping population was also observed by Xiao et al. (2011a), Cheng et al. (2012), Ye et al. (2012), Li et al. (2018), and Cao et al. (2020). The contrast observed between the parents for spikelet fertility was noteworthy in the present study, because this supersedes earlier reported contrasts such as those between Azucena and Bala (Jagadish et al. 2010), Xiushui 09 and IR2061 (Cheng et al. 2012), IAPAR-9 and Liaoyan 241 (Li et al. 2018), and between IR64 and Koshihikari (Seo et al. 2019). However,

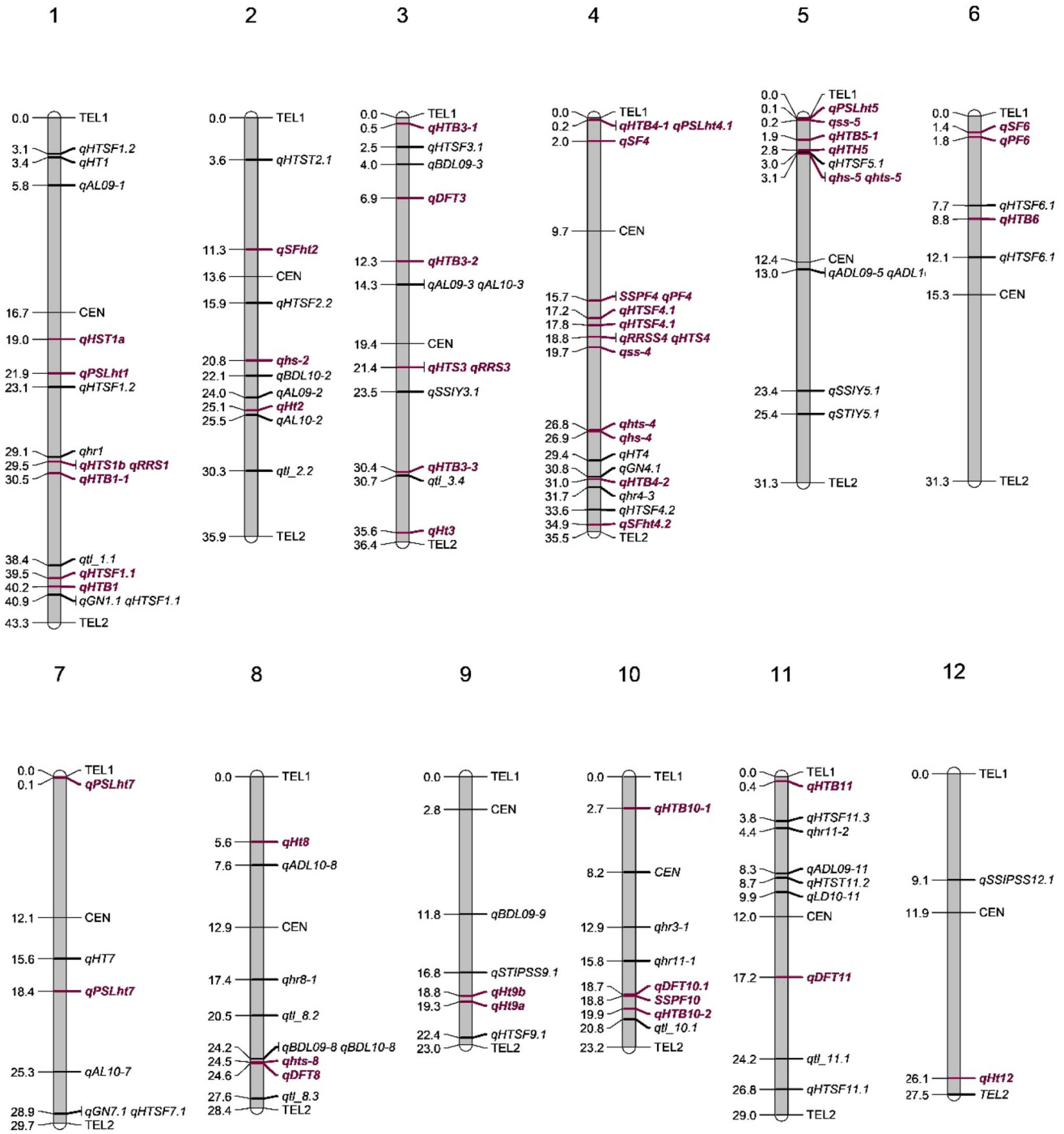

Fig. 5 The physical positions (in Mb) of various QTLs previously reported for reproductive stage heat stress tolerance in rice. The QTLs analysed in the present study using SSR markers are highlighted in purple colour. TEL1 and 2 indicates telomeres and CEN represents centromere. The additional details of the QTLs are provided in supplementary table S1

superior contrasts than that in this study was also reported between cultivars such as 996 and 4628 (Xiao et al. 2011a), Sasanishiki and Habataki (Zhao et al. 2016; Zhu et al. 2017), N22 and IR64 (Ye et al. 2012) and between R53 and HHT4 (Cao et al. 2020). Moreover, the mean spikelet fertility of 77% under stressed conditions exhibited by NL44 was higher than that was observed by Bahuguna et al. (2015) under similar temperature regime (38 °C) in the same cultivar. Also, the higher spikelet fertility among the RILs than that was reported in many of the previous studies indicated that NL44 can act as a potential donor for this trait. These observations were further corroborated by

Table 4 Summary of parent polymorphism survey carried out using reported QTL linked SSR markers reproductive stage heat stress tolerance

Chromosome no.	Number of QTL linked markers	Number of polymorphic markers[a]
1	11	3 (27%)
2	7	2 (29%)
3	22	5 (23%)
4	24	7 (29%)
5	10	3 (30%)
6	9	2 (22%)
7	4	1 (25%)
8	9	2 (22%)
9	4	1 (25%)
10	8	2 (25%)
11	6	2 (33%)
12	2	1 (50%)
Total	116	31 (26.7%)

[a]The percentage of polymorphic markers are given in parenthesis

Table 5 Markers showing significant association with the respective traits revealed through single marker analysis

Marker	SF_S			$STI_{(sf)}$			Linked QTL	
	PVE	Probability	Additive effect	PVE	Probability	Additive effect	QTL ID	Reported PVE
RM3355	5.7	0.016	− 3.03				*qhs2*	6.4
RM401	3.8	0.037	2.69	3.8	0.0261	0.02	*qSF4*	10.3
RM5687	4.8	0.026	3.05				*SSPF4, qPF4*	21.3
RM6394				6.9	0.0027	0.03	*qPSLht7*	–
RM6673	3.6	0.037	2.59				*qHTB10-2*	–
RM7283	3.3	0.047	2.49				*qHTB11*	–

SF_S, spikelet fertility under stress; $STI_{(sf)}$, stress tolerance index for spikelet fertility; PVE, percent variation explained (%)

the estimated STIs for spikelet fertility and grain yield. These indices were previously utilized by Shanmugavadivel et al. (2017) for mapping reproductive stage heat stress tolerance.

One of the most interesting observations in this study was the stability of pollen fertility in NL44 and PB1 under RSHS. A similar pattern was also observed among the RILs. This implies that pollen fertility *per se* was not the factor that led to spikelet sterility and the consequent yield reduction in PB1 under RSHS. There are several factors that define successful fertilization and grain development in rice, that are attributable to high temperature tolerance/sensitivity. Such factors include anther dehiscence, pollen desiccation, poor pollen germination, drying up of stigma as well as embryo abortion. Some other impairment on the male reproductive system could also not be ruled out (Jagadish et al. 2010, 2015). The I_2-KI staining method, that is routinely used to estimate pollen fertility, captures the starch accumulation in the pollen grains which is essential for the germination on the stigma. Nevertheless, other pollen traits such as pollen coat, composed of other biochemical compounds such as lipids and proteins, also play a vital role in pollen viability. Basing pollen viability solely on starch-based iodine staining may therefore be a false positive for fertility and may lead to misleading conclusions (De Storme and Geelen 2014). Therefore, rather than pollen fertility, spikelet fertility must be considered as the most reliable trait for determining RSHS response. Besides, spikelet fertility has been found to have an important bearing on grain yield under heat stress and hence qualifies as an important yardstick to judge genotypes for their heat stress tolerance (Prasad et al. 2006). Furthermore, we found that the spikelet fertility, grain yield and the derived stress tolerance indices followed normal distribution, qualifying these traits for mapping because of their true quantitative nature of inheritance, as reported earlier (Jagadish et al. 2010; Cheng et al. 2012; Ye et al. 2012, 2015a; Zhao et al. 2016; Shanmugavadivel et al. 2017; Zhu et al. 2017) meriting a QTL mapping study to identify the concerned genomic regions in further studies.

As previously mentioned, there has been studies that have mapped QTLs for RSHS tolerance in rice. Therefore, this warrants a pre-survey of the reported QTLs on any identified donor source before declaring it to be novel. BSA has been exemplified as a simple and rapid method to identify markers putatively linked to the target genomic regions when parents are highly contrasting for the trait of interest (Michelmore et al. 1991). This approach not only saves time but also saves resources that would otherwise go into mapping exercise. Propelled by next-generation sequencing (NGS) technologies, it has emerged as a robust alternative and method of choice for mapping studies in various crops (Takagi et al. 2013a, b; Zou et al. 2016). Since the parents used in this study, showed adequate contrast for RSHS response, we have employed BSA as the prefatory approach towards excluding the already known QTLs, if any. However, the polymorphic markers that defined 27% diversity between the parental genomes failed to strike any relation with the markers and the traits, with which the bulks were constituted such as grain yield reduction and spikelet fertility. It may be argued that the number of SSR markers used were limited, and the complexity of the trait itself (reproductive stage heat tolerance) might have been the reason for the lack of any putative association in BSA. This indicated that there may not be a specific set of markers or genomic regions among the ones targeted in the present study, that defined the RSHS response among the parents, hence the bulks failed to expose them.

In order to identify any QTLs that are involved in the stress response, we have run the polymorphic markers on all the RILs, leading to identification of six minor QTLs. The corresponding linked markers were distributed on chromosomes 2, 4, 7, 10 and 11 and were reported associated to QTLs, *viz*., *qhs2*, *qSF4, SSPF4, qPF4*, *qPSLht7*, *qHTB10-2* and *qHTB11,* respectively. However, the cumulative effect of these minor QTLs when exposed to RSHS also could not explain the contrast between NL44 and PB1, as well as between the bulks. For instance, the cumulative additive effect of these minor QTLs on NL44 was 7.86% for spikelet fertility, while the contrast between NL44 and PB1 was 52.4%. Therefore, the minor QTLs that were already identified from other genetic backgrounds could explain only 30% of the contrast between NL44 and PB1, leading us to conclude that NL44 must be possessing additional regions that may contribute to its high RSHS tolerance. Divulgence of these novel QTL regions requires exploration of genome wide variation between PB1 and NL44.

Among the numerous QTLs reported for RSHS tolerance, two major QTLs, *qHTSF1.1* and *qHTSF4.1* located respectively on chromosome 1 and 4, were reported from N22 for spikelet fertility (Ye et al. 2012). Notably in this study, the markers RM431 and RM5757 linked to *qHTSF1.1* and *qHTSF4.1* respectively (Ye et al. 2012, 2015a, b; Vivitha et al. 2017), were monomorphic between NL44 and PB1. Both the genotypes possessed the alternate allele for these markers in contrast with N22, emphasizing that heat stress tolerance in NL44 could be different from that of N22. However, we could identify six minor QTLs, associated with markers related to seven putative QTLs already reported. Among these, the marker RM3355 was reported to be associated with spikelet fertility (*qhs2*) under high temperature (Zhao et al. 2006). Another marker, RM401 linked to *qSF4* located on chromosome 4 at physical location of 2.03 Mb (Cheng et al. 2012) was found associated to both spikelet fertility as well as STI in this study. This QTL located away from one of the robust QTLs, *qHTSF4.1* (17.69 Mb), could not be therefore implicated as associated to *qHTSF4.1*. Similarly, RM5687 that was reported associated with seed set percentage, *SSPC4* (Xiao et al. 2011a) and also to the QTL, *qPF4* for pollen fertility (Xiao et al. 2011b), was also located on chromosome 4. A marker on chromosome 7, RM6394 that was reportedly linked to pollen shedding level under heat stress (*qPSLht7*) (Zhao et al. 2016) was identified for spikelet fertility in our study. Further, another linked marker to spikelet fertility RM6673, was earlier reported to be associated with two QTLs (*qHTB10-2* and *qDFT10.1*) from two separate studies (Zhu et al. 2017; Zhao et al. 2016). Finally, the marker RM7283, that was associated with *qHTB11* as reported for spikelet fertility by Zhu et al. (2017) was also found linked to the same trait among the NL44 RILs. Thus, we could demonstrate that the major genomic regions conferring the RSHS tolerance in NL44 may be different, to a larger extent, from those reported in previous studies, and would act in tandem with few minor QTLs to display the tolerance.

Conclusion

In conclusion, the present study established that NL44 may be a plausible novel donor for RSHS tolerance in rice. Among the traits studied, the spikelet fertility has emerged as the most ideal trait for assessing the RSHS response. The SSR markers linked to the reported QTLs were found mostly ineffective in discerning the tolerance conferred by NL44, except for six minor ones. Since the percentage of total variation accounted for by these minor QTLs was low, and the cumulative additive effect of these QTLs was inadequate to explain the contrast between NL44 and PB1, we conclude that NL44 harbour novel genomic regions conferring RSHS tolerance. The total tolerance response of NL44 could be driven by the complementary effects of these minor QTLs with yet to be mapped genomic regions.

Identification of these novel regions require extensive genome wide studies, so that NL44 can be used as a potential donor for breeding RSHS tolerant climate resilient cultivars for the future.

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Authors contributions GKS and AKS conceived the idea and framed the research plan. RKT carried out the research work and prepared the manuscript. NM and PKB assisted in the execution of field trials. MACR gave inputs for the molecular marker analysis. DG, DP, PK and VP assisted in molecular work. VKK and ERK assisted in data analysis. MP shared donor seed material. GKS and HB improved the manuscript. AKS provided overall guidance in each of these activities.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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