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Research article

Development and validation of markers to improve heat tolerance in flowering stage of Laos elite rice cultivar

Sontayar Phethavone[a,b], Thanyalak Jampa[b], Cécile Grenier[c,d], Julien Frouin[c,d], Watchareewan Jamboonsri[e], Chanate Malumpong[b], Phetmanyseng Xangsayyasane[a], Theerayut Toojinda[e], Jonaliza Lanceras Siangliw[e,*]

[a] *Rice Research Center, National Agriculture and Forestry Research Institute, Ministry of Agriculture and Forestry, Vientiane Capital, P.O. Box 7170, Laos*

[b] *Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*

[c] *Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), UMR Amélioration génétique et adaptation des plantes méditerranéennes et tropicales (AGAP), Montpellier F-34398, France*

[d] *AGAP, Univ Montpellier, CIRAD, Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRA), Montpellier SupAgro, Montpellier F-34398, France*

[e] *National Center for Genetic Engineering and Biotechnology (BITOEC), NSTDA, Pathum Thani 12120, Thailand*

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Abstract

Importance of the work: Rice is sensitive to high temperatures in almost all its growth stages, with temperature being a major factor causing poor seed-setting rates.

Objectives: To validate molecular markers linked to two quantitative trait loci associated with high-temperature spikelet fertility, namely *qHTSF1.1* and *qHTSF4.1*

Materials & Methods: F_2 lines were grouped as H1 and H2 with the HXBF2 allele at *qHTSF1.1* and the N22 and HXBF2 alleles at *qHTSF4.1*, respectively, while groups H3 and H4 had the N22 allele at *qHTSF1.1* and the N22 and HXBF2 alleles at *qHTSF4.1*, respectively. Stressing was done at 40–45°C for 6 hr from the booting to the harvesting stage. Pollen viability and spikelet fertility were used to assess tolerance to heat.

Results: Parental lines were significantly different for pollen viability and spikelet fertility under heat stress. N22 was not affected by high temperatures, while HXBF2 showed a severe reduction in pollen viability (66%). N22 maintained 76% spikelet fertility under heat stress, making it moderately tolerant to heat stress with an 11% reduction against the 25% reduction in spikelet fertility of HXBF2. Under heat stress, the F_2 lines of groups H1 and H3 had similar pollen viability scores to N22, while only the H1 group could be classified as heat-tolerant, maintaining good spikelet fertility.

Main finding: The efficiency of marker-assisted selection was confirmed in the F_2 3-way population phenotyped for pollen viability and spikelet fertility under heat stress conditions. The molecular markers, particularly *qHTSF4.1*, should be very useful for marker-assisted selection breeding.

* Corresponding author.
E-mail address: jonaliza.sia@biotec.or.th (J.L. Siangliw)

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Introduction

Before 2030, crop production must be increased by 50% to feed the unavoidable growth in the world's population (Tomlinson, 2010). However, it has been predicted that the average temperature will rise by 2–3°C over the next 30–50 yr (Hatfield and Prueger, 2015). Such a global climate change will result in worldwide challenges because higher temperatures are expected to have negative impacts on agricultural productivity through reduced crop production (Jagadish et al., 2010a; Wahid et al., 2007; Root et al., 2003), generating added pressure to sustain global food security in the future (Pradhan and Prasad, 2015).

Rice (*Oryza sativa* L.) is one of the world's leading crops, particularly in Asia, with its importance increasing in Africa and Latin America (Manigbas et al., 2014). Rice is grown in irrigated systems, with additional production from rainfed and upland cropping systems. At present, drought and high temperatures are becoming common problems in many rice areas (Fahad et al., 2019).

In most rice-producing areas, the temperature variation is close to optimum at 28°C and 22°C for day and night mean temperatures, respectively (Das et al., 2014). At temperatures in the range 27–32°C, rice is still able to maintain normal growth; however, day temperatures above 32°C have negative effects on all stages of the rice growth and development (Aghamolki et al., 2014). High temperatures affect physiological processes, such as stomatal opening, photosynthesis and growth, as well as causing early physiological maturity and shortening the growth period (Prasad et al., 2006; Wassmann et al., 2009; Krishnan et al., 2011). Jagadish et al. (2007) reported that 33°C was a critical factor during the flowering stage. Temperatures higher than 35°C for just 1 hr during flowering induced a reduction in pollination and increased spikelet sterility, which severely reduced the grain yield (Satake and Yoshida, 1978; Matsui et al., 1997). Furthermore, high temperatures during flowering makes the pollen grains sticky, which, in turn, induces choking of grain due to basal dehiscence, with pollen remaining in the anthers (Matsui et al., 2005).

Exposure of flowering spikelets to high temperatures results in the failure of anther dehiscence and reduced numbers of germinating pollen grains on the stigma (Satake and Yoshida, 1978; Matsui et al., 2001; Matsui and Omasa, 2002; Jagadish et al., 2010a). Damage to rice productivity is expected when the maximum temperature reaches 40°C in the flowering stage (Hasegawa et al., 2011). Spikelet sterility relates to yield reduction in the field and potential yield decline (Kim et al., 1996; Erda et al., 2005; Oh-e et al., 2007). In addition, the floral buds are unable to produce carbohydrates under heat stress (Zinn et al., 2010). The effects of high temperatures are prominent in tropical Asia (Osada et al., 1973; Van Zonneveld et al., 2009; Yasukawa and Uchida, 2018) and Africa (Matsushima et al., 1982). Consequently, based on the conditions cited above, genetic improvement of heat stress-tolerant rice varieties is one of the best options for maintaining rice productivity (Kilasi et al., 2018).

Rice genetic resources tolerant to high temperatures have been identified in both the *indica* (Matsui et al., 1997) and *japonica* subspecies (Matsui et al., 2001). There are two ways to mitigate heat-induced spikelet sterility: create cultivars that shed more pollen grains or develop pollen grains that can germinate at high temperatures. Variety Nagina 22 (N22) is an Indian *aus*-type landrace that has heat tolerance and has been mostly used as a donor of heat tolerance in the reproductive stage (Satake and Yoshida, 1978; Mackill et al., 1982; Prasad et al., 2006; Jagadish et al., 2010a).

Quantitative trait loci (QTL) for rice heat tolerance in the flowering stage have been mapped on all chromosomes except on chromosome 7 based on various rice populations and high temperature treatment methods (Cao et al., 2003; Chen et al., 2008; Jagadish et al., 2010b; Xiao et al., 2011; Ye et al., 2015). Ye et al. (2012) used 384 single-nucleotide polymorphism (SNP) markers to identify QTL for heat tolerance in the flowering stage of a rice population derived from a cross between IR64 and N22 using F_2 and BC_1F_1 populations. Two QTL, explaining 12.6 and 17.6% of phenotypic variance, were detected on chromosomes 1 and 4, respectively. The QTL *qHTSF1.1* on chromosome 1 was located between SNP markers id1023892 and id1024836 at about 39.55 Mb. The QTL *qHTSF4.1* was located on chromosome 4 between SNP markers id4005120 and id4005867 at about 17.69 Mb. Unexpectedly, the tolerant allele of *qHTSF1.1* was from the susceptible parent IR64, while the allele contributing to *qHTSF4.1* was from the tolerant parent N22 (Ye et al., 2012). Ye et al. (2015) reported that the N22 allele at *qHTSF1.1* would even counteract the positive effect conferred by the N22 allele at *qHTSF4.1* in an IR64 × N22 population. In addition, *qHTSF4.1* was reported as one of the most consistent QTL in populations developed from crosses involving the heat tolerant genotypes N22 or Giza 178 (Ye et al., 2012). The effectiveness of *qHTSF4.1* was exposed in selected BC_2F_2 progenies from the same IR64 × N22 cross. The plants with *qHTSF4.1* showed significantly higher spikelet fertility than other genotypes and confirmed that both *qHTSF1.1* and *qHTSF4.1* are very important QTL for increasing spikelet

fertility under high temperature. Ye et al. (2012) reported that having the IR64 and N22 alleles in *qHTSF1.1* and *qHTSF4.1*, respectively, increased the spikelet fertility by 64% compared to just having the IR64 allele in both QTL and by 93% when the N22 allele was present in both QTL. The interaction between *qHTSF1.1* and *qHTSF4.1* revealed that in the presence of *qHTSF1.1* alone, there may be a slight increase in spikelet fertility without the presence of *qHTSF4.1*; however, *qHTSF4.1* could significantly increase spikelet fertility under high temperature only when combined with *qHTSF1.1*. Lafarge et al. (2017) conducted genome-wide association studies (Genome-wide association studies) using 167 rice varieties. The expected QTL were found on chromosomes 1 and 4, with *qHTSF1.1* (30,476,158 bp) containing Os01g53020 (heat shock protein Dunaj) and *qHTSF4.1* (17,876,533 bp) containing Os04g29960 (OsWAK43-OsWAK receptor-like protein kinase) and Os04g29990 (OsWAK44-OsWAK receptor-like protein kinase), respectively. While the importance of these two QTL has been reported and confirmed in the aforementioned studies, there are still doubts concerning the source of the favorable allele. Elucidating whether the N22 allele could have a negative effect at *qHTSF1.1* is a valid outcome prior to the introgression of heat tolerance in Laotian rice.

The current project focused on elite cultivars in the Lao People's Democratic Republic (Laos) which need to increased tolerance to global warming and high day temperatures, notably during the pollination period. Rice in Laos requires various key traits, such as tolerance to submergence, pest and disease resistance and grain quality. These characteristics have already been introgressed in Homxebangfai 2 (HXBF2), a variety largely grown in Laos. A crossing scheme with RGD13297-124-8-2-MAS3-MAS1 (GCP_Laos-TDK1, hereafter named GCP) was performed to introduce resistance to Xanthomonas, while more recently, there has been a cross with N22 to improve spikelet fertility (seed setting) under heat stress.

The three parental genotypes in the current study were investigated to identify 23 SNPs among the genotypes in the regions harboring the two QTL (14 on chromosome 1 and 9 on chromosome 4) and four SNP markers were designed to follow the introgression of the respective QTL. The F_2 population derived from the three-way cross between HBXF2, GCP and N22 was screened under conditions of 40–45°C for 6 hr of day temperature during the full duration of the booting to the harvesting stages to score spikelet fertility under high-temperature stress. The objective was to confirm new heat tolerance markers by observing the phenotypes of pollen viability and spikelet fertility as indices of heat tolerance in an F_2 population. Once the markers have been validated, it should be possible to select lines for the development of new heat tolerant rice varieties.

Materials and Methods

Plant materials

HXBF2 is an aromatic, non-glutinous rice with good eating quality due to its low amylose content. It has submergence tolerance but with low percentage of seed setting under high-temperature conditions. GCP is an aromatic, submergence tolerant, blast and bacterial blight resistant variety but it is susceptible to heat stress. N22 has a deep root, showing resistance to drought and is tolerant to heat stress. IT has been used in breeding programs to improve drought and heat tolerance. SNPs markers were investigated to hasten the development of HXBF2 with improved heat tolerance.

The elite parental line HXBF2 was crossed with two donor parents (GCP and N22) to generate a population improved for bacterial blight resistance and that could be segregated for spikelet fertility. Seeds of HXBF2 and GCP were available at the National Agriculture and Forestry Research Institute (NAFRI) while N22 was accessed from the International Rice Research Institute gene bank. The first cross involved HXBF2 and GCP and the resulting F_1 was crossed with N22 to incorporate heat tolerance. The F_1 lines from the 3-way cross were allowed to self-pollinate to produce F_2 seeds that were genotyped using four heat tolerance markers and validated for heat tolerance (Fig. 1). The 46 F_2 plants, the three parental lines and Sinlek, a susceptible check, were used in heat tolerance screening.

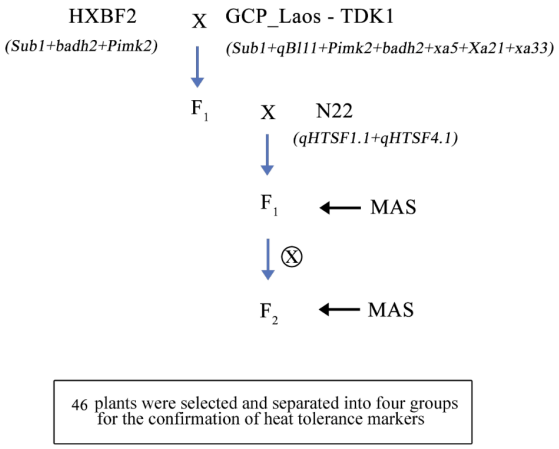

Fig. 1 Breeding scheme in developing F_2-HXBF2 with bacterial blight resistance donor (GCP) and heat tolerant N22 based on marker-assisted selection (MAS)

Heat tolerance markers

Molecular markers for *qHTSF1.1* and *qHTSF4.1* were searched on chromosome 1 between 38.2 Mbp and 39.7 Mbp and on chromosome 4 in the region 17.6 Mbp to 18 Mbp. Four KASP (Kompetitive Allele Specific) polymerase chain reaction (PCR) markers from a set of markers established by the Integrated Breeding Platform (https://www.integratedbreeding.net) were selected for their polymorphism among the parental lines involved in the development of the populations (Table 1). There were three markers for *qHTSF1.1* and one for the *qHTSF4.1* region.

All KASP markers were ordered from a primers synthesis company for high-throughput genotyping, following the instruction of the KASP mix providers.

DNA extraction and polymerase chain reaction amplification

Genomic DNA from leaf tissues was extracted using the DNA Trap (DNA technology laboratory) method. The DNA samples were amplified based on PCR using specific markers (Table 1) to detect lines carrying the target alleles. A mixture of 1.5 µL master mix/sample (Biosearchtech™), 0.045 µL assay/sample (Biosearchtech™), and 1.455 µL dH_2O were mixed in a 2 µL Eppendorf tube and 3 µL/sample of the mixture was added to the DNA (concentration of 75 ng/sample) and PCR was initiated by denaturing the DNA at 94°C for 2 min., followed by 27 cycles of 95°C for 30 s, 55°C for 40 s and 72°C for 1 min for the denaturation, annealing and extension, respectively. The PCR products were analyzed using QuantStudio 12K flex (Applied Biosystems™).

Once genotyped with the four SNP markers, the F_2 plants were separated into four groups based on the allele combinations at the two QTL (Table 2).

Phenotyping of F_2 (3-way) population

The F_2 plants together with their parents (HXBF2, GCP and N22) and susceptible check Sinlek were considered for heat tolerance screening. Eight plants per variety for parents and a check and 46 F_2 (14 for H1, 14 for H2, 8 for H3 and 10 for H4, as shown in Table 2) plants, were grown singly in plastic pots (20 cm width, 20 cm length and 30 cm height) filled with clay loam soil. The pots were placed under field conditions from May to September 2020 at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. For the heat stress treatment, at booting stage, when the elongating boot reached 5 cm between leaf sheath and flag leaf (R2: collar formation on the fag leaf), the plants were transferred to a controlled greenhouse equipped with a continuous ventilation system in which the day temperature was maintained at 40–45°C for 6 hr (from 1000–1600 hours) and the night temperature was maintained at the outside ambient temperature (25–30°C; mean 26°C). All tillers with a 5 cm boot in both the control and heat stress treatments were tagged and the date was recorded. Flowering at 50% was recorded. Within each group, (either the F_2 or the varieties), one-half of the plants were transferred to the heat stress condition while the other half were left in the open field to serve as the control treatment. The plants were exposed to these specific conditions until harvested.

Table 1 Single-nucleotide polymorphism markers developed and validated to follow the introgression of heat tolerance into HXBF2

Chr	Position	Locus	N22	HXBF2	GCP
1	38,200,000	*qHTSF1.1*			
1	38,632,196	K_id1024503	Allele T	Allele A	Allele A
1	39,136,724	K_id1024836	Allele C	Allele G	Allele G
1	39,369,209	K_id1024973	Allele G	Allele A	Allele A
1	39,700,000	*qHTSF1.1*			
4	17,600,000	*qHTSF4.1*			
4	17,906,798	K_id4005212	Allele C	Allele T	Allele T
4	18,000,000	*qHTSF4.1*			

Table 2 Groups of allele combinations and number of plants per group used in heat tolerance screening for marker validation

Group	qHTSF1.1			*qHTSF4.1*	Plant number
	K_id1024503	*K_id1024836*	*K_id1024973*	K_id4005212	
H1	HXBF2	HXBF2	HXBF2	N22	14
H2	HXBF2	HXBF2	HXBF2	HXBF2	14
H3	N22	N22	N22	N22	8
H4	N22	N22	N22	HXBF2	10
Total					46

Microscopic observations

The spikelets sampled for pollen viability from the plants treated with heat stress and for the control conditions were selected from the second or third branch from the top on the primary rachis in the central position. Anthers were collected from 0700–0800 hours (to avoid anthesis) from five random spikelets per panicle. The spikelets were fixed in 70% ethanol and stored at 4°C. An I_2-KI solution (1%; 40 µL) was added on a glass slide and six anthers from the spikelets were placed on the glass slide in the I_2-KI solution. The anthers were gently crushed to release the pollen onto the glass slide and then covered with a cover slip. Pollen was mounted on slides and was observed and photographed under a microscope (Leica DM750; 10×10 magnification). Pollen grains that stained black were considered viable, while those stained yellow or light-colored were counted as sterile. Pollen viability (PV) was counted as the ratio of number of stained pollen-to-total number of pollen grains and was expressed as a percentage (Prasad et al., 2006), based on Equation 1:

$$\text{Pollen viability} = \frac{\text{Number of pollen grains stained black}}{\text{Total number of pollen grains}} \times 100 \quad (1)$$

Spikelet fertility

To evaluate the spikelet fertility, the number of filled grains and the number of unfilled grains were counted separately from three panicles harvested from each of the F_2 line, the parents and the check. The percentage of seed setting was used to determine the spikelet fertility (SF), based on Equation 2:

$$\text{Spikelet fertility} = \frac{\text{Number of filled grains}}{\text{Total number of spikelets}} \times 100 \quad (2)$$

The Standard Evaluation System for Rice or SES (International Rice Research Institute, 2013) was used to score for the percentage spikelet fertility as the criterion for selecting heat tolerant rice. The SES scoring applied was: 1 = highly fertile (> 80%), 3 = fertile (61–89%), 5 = partly sterile (50–74%), 7 = highly sterile (< 50% to trace) and 9 = 0.

Data analysis

Data on all parameters (pollen viability and spikelet fertility) were analyzed using one-way analysis of variance per group and among groups using the GenStat v.22 software. Correlations and graphs were created using the R-CRAN packages statistical program version 3.6.1 (R Core Team, 2019). Significance was tested at $p < 0.05$ level.

Results

Daily mean temperature for field and heat stress in greenhouse

The booting date in each group started on 17 July 2020 and 50% flowering started on 27 July 2020. The panicles were harvested during the last week of August to the first week of September 2020 for both the heat stress and control treatments. The daily mean temperature at the booting stage during the day was 35–43°C and 27–33°C during the night in the greenhouse for the heat stress treatment and 25–30°C during the day and 25–28°C during the night for the control (natural field) conditions. The daily mean temperature for the heat-stress condition in the greenhouse was always higher than for the control during both the day and night (Fig. 2).

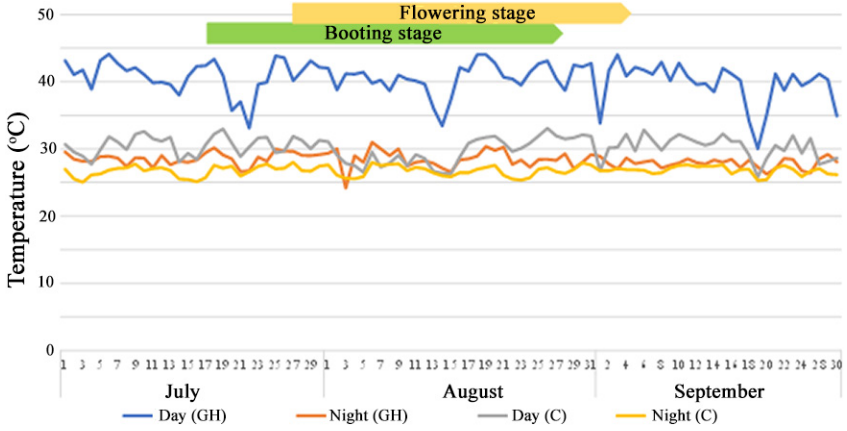

Fig. 2 Daily mean temperatures during day and night in field (C; control) and greenhouse (GH; stress conditions) for booting to harvesting stages during heat stress experiment

Percentage of pollen viability under control and heat stress conditions

The mean pollen viability (PV) of the parents, checks and groups of F_2 lines for the control conditions was high, ranging from 79% in H4 to 98% in GCP (Table 3). The mean PV of lines/varieties tested under either the control or heat stress conditions were significantly different. For the control conditions H4 had significantly lower PV (79%) than any other F_2 group or variety. Under heat stress, N22 had the highest PV (95%) and was significantly higher than HXBF2 (33%). Among the groups of F_2 lines, H1 had the greatest PV under HS (95%) followed by H3 (91%), while the PV of the H1 group was not significantly different from N22 (Table 3). On the contrary, H2 and H4 had the lowest PV values under HS (32% and 23%, respectively) that were comparable to those of HXBF2 for H2 and the susceptible parent, GCP for H4. The parental lines HXBF2 and GCP had similarly high PV reductions under the HS conditions (66% and 78%, respectively). These drops in PV were comparable to those of Sinlek, the susceptible check, which had viable pollen as low as 25% under the HS conditions. The mean PV under heat stress decreased significantly in H2 and H4, with almost the same order of magnitude (around 70%) as for the susceptible parents and check, while H1 and H3 showed only 0.6 and 2.5% reductions. These two groups of F_2 had comparable reductions in PV to N22.

Percentage of spikelet fertility under control and heat stress conditions

The percentage of spikelet fertility (SF) under the control conditions ranged from 62% in H4 to 85% in N22 and was significantly different among the group of lines/varieties tested. Among the parental lines, there was no significant difference in SF for N22 and GCP, with values around 85% and 84% (Table 4). On the other hand, H4 had a significantly lower SF than any other F_2 group (62%) and was even lower than the susceptible check Sinlek (69%). Under heat stress, SF was also significantly different among the F_2 groups and the parental lines (Table 4). N22 had the highest percentage of fertile spikelets (76%), while both HXBF2 and GCP had 59% fertile spikelets—much higher than for Sinlek, the susceptible check (24%). The F_2 groups were clustered in three sets: H1 with high SF (75%) comparable to N22; H4 with low SF (around 50%); and H2 and H3 with intermediate SF (58–62%), which was similar to the two susceptible parents. The reduction in percentage of SF due to high temperatures was significant and encountered in all genotypes except for those of F_2 in group H1. The percentage of SF among the F_2 groups declined by 18%, 18% and 20% in H2, H3, and H4, respectively, (Table 4). The degree of SF loss reported in these three groups of F_2 was greater than for N22 (11%), lower than the susceptible parents (25% and 29%) and much lower than the decrease in the susceptible check (65%).

Table 3 Mean pollen viability under heat stress condition compared with control conditions, and a decrease in pollen viability of F_2, parents, and reference lines

Group	Control condition (Mean ± SD)	Heat stress condition (Mean ± SD)	% Reduction
H1	95.2 ± 1.4^{cde}	94.6 ± 0.8^{d}	-0.6
H2	94.9 ± 1.4^{cd}	31.7 ± 1.6^{b}	-66.6
H3	93.2 ± 1.8^{bc}	90.9 ± 0.7^{c}	-2.5
H4	79.4 ± 1.4^{a}	22.8 ± 1.7^{a}	-71.3
N22	95.7 ± 1.4^{cde}	95.7 ± 0.2^{d}	0.0
HXBF2	96.6 ± 0.9^{de}	32.7 ± 1.3^{b}	-66.1
GCP	97.6 ± 0.4^{e}	21.8 ± 3.7^{a}	-77.7
Sinlek	91.3 ± 0.8^{b}	25.2 ± 1.7^{a}	-72.4
Mean	93.0	52.9	
p-value	1.29e-08***	< 2e-16***	

Mean ± SD superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Table 4 Mean spikelet fertility under heat stress condition compared with control conditions, and a decrease in pollen viability of F_2, parents, and reference lines

Group	Control condition (Mean ± SD)	Heat stress condition (Mean ± SD)	%Reduction
H1	72.5 ± 1.9^{bc}	74.8 ± 1.7^{d}	3.2
H2	70.7 ± 2.6^{b}	58.2 ± 1.8^{c}	-17.7
H3	76.0 ± 1.8^{cd}	62.2 ± 3.6^{c}	-18.2
H4	61.9 ± 2.4^{a}	49.5 ± 3.3^{b}	-20.0
N22	85.3 ± 2.0^{f}	76.2 ± 2.6^{d}	-10.7
HXBF2	78.6 ± 2.6^{de}	58.9 ± 4.4^{c}	-25.1
GCP	83.5 ± 1.1^{ef}	59.2 ± 1.7^{c}	-29.1
Sinlek	69.4 ± 2.0^{b}	24.4 ± 3.5^{a}	-64.8
Mean	73.9	59.0	
p-Value	1.47e-12***	<2e-16***	

Mean ± SD superscripted with different lowercase letters are significantly ($p < 0.05$) different; Negative % reduction denotes decrease of spikelet fertility (SF) under heat stress, while positive % reduction denotes increase in SF under heat stress.

Relationship between percentage of pollen viability and percentage of spikelet fertility under control and heat stress conditions

Highly significant correlations between PV and SF were found under the control (correlation coefficient (r) = 0.53; $p < 0.001$) and heat stress (r = 0.65; $p < 0.001$) conditions (Fig. 3). Under the control conditions, almost all groups of F_2 lines clustered together with N22, the susceptible parents and the check, with them all characterized being by high PV and SF values. The only exception was H4 that had the lowest PV (80%) and SF (62%). Under the HS conditions, the clustering of the genotypes was clear, with the tolerant N22 having extremely high PV and SF values, close to the H1 and H3 groups. In contrast, the susceptible check had low PV (25%) and SF (25%) values. The third group contained the susceptible parents (HXBF2, GCP) and the H2 and H4 groups. While H3 and the susceptible parents had comparable SF values, they clustered differently on the biplot because H3 had a significantly higher PV value.

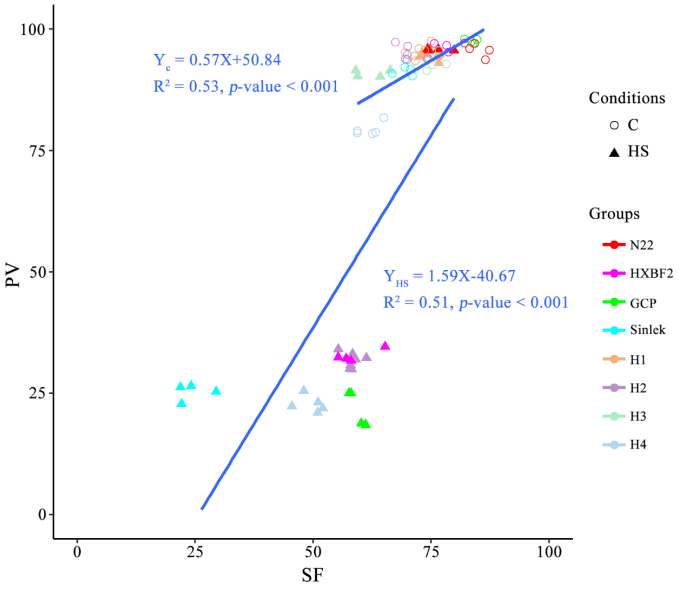

Fig. 3 Linear correlations (shown by straight lines) between pollen viability (PV) and spikelet fertility (SF) under control (C; open circles)) and heat stress (HS; filled triangles) conditions, where R^2 = coefficient of determination, Y_{HS} = PV and X = SF

Discussion

Two QTL for heat tolerance (*qHTST1.1* and *qHTST4.1*) identified by Ye et al. (2012) were considered in an introgression breeding scheme using marker-assisted selection. In a study conducted by Xiao et al. (2011), a QTL for heat tolerance was found on chromosome 4, bounded by markers RM5687 and RM471. Using the BLAST tool with the National Center for Biotechnology Information database, RM5687 and RM471 were aligned with a Nipponbare sequence from 15,914,227 bp to 18,997,169 bp and therefore, encompassing the *qHTSF4.1* identified by Ye et al. (2012). Xiao et al. (2011) mapped pollen fertility on the same region of chromosome 4. Other studies (Ye et al., 2015; Lafarge et al., 2017), showing the genomic positions of heat tolerance QTL on chromosomes 1 and 4 related to spikelet fertility, indicated the importance of these positions for heat tolerance in rice and this information was used in the current study in developing molecular markers in introgression of those QTL to the HXBF2 genetic background.

To evaluate the efficiency of markers developed for the introgression of *qHTST1*.1 and *qHTST4.1* in Laos elite germplasm, an F_2 population was developed between HXBF2 and N22 and screened for pollen viability and spikelet fertility under high day temperatures. In the greenhouse experiment, heat stress was applied from the booting stage until harvest, with day temperatures reaching 40–45°C during 1000–1600 hours. Under this condition of extreme temperatures, N22 was able to produce a high percentage of fertile spikelets. Cheabu et al. (2018) used temperatures similar to the current conditions and reported similar observations concerning the high tolerance of N22 and the extreme heat sensitivity of the susceptible check, Sinlek. The repeatability of results guaranteed that the applied stress protocol produced results that were confirmable and an indication that the F_2 introgression lines were properly screened for heat tolerance.

In general, rice is the most susceptible crop to heat stress during the flowering stage. Extremely high temperatures in the greenhouse significantly decreased pollen viability and spikelet fertility in the current study. Notably the PV under the control conditions was high (79–98%) while SF (62–85%) was lower than PV for the same conditions. The low SF observed may have been due to the field conditions (Fig. 2), where the temperature was many times higher than 30°C. Thuy et al. (2020) identified that at 33°C, pollen viability decreased, resulting in lower spikelet fertility. The current observations showed that under heat stress the PV and SF values were for

H1 (HXBF2|N22), with a similar tolerance level to that of N22. The H3 (N22|N22) group had some degree of tolerance, but only in terms of PV, with values slightly lower than those of N22 and H1, while its SF scores were close to those of HBXF2 and the H2 group. The H2 (HXBF2|HXBF2) group had similar PV and SF values as the parent HBXF22, while the values for H4 (N22|HXBF2) were worse than for HXBF2 and similar to GCP. It could be concluded that the sole introgression of the N22 allele at *qHTSF4.1* was able to improve heat tolerance in susceptible HXBF2 and the marker at locus K_id4005212 was effective in selecting rice with heat tolerance. Ye et al. (2012) reported that there was an interaction between *qHTST1.1* and *qHTST4.1*, resulting in a significant increase in spikelet fertility in plants carrying the appropriate alleles for those QTL. This finding was consistent with the current results. The correlation of pollen viability and spikelet fertility supported the findings in H1 and N22 that high pollen viability resulted in high spikelet fertility. Thuy et al. (2020) also found a positive correlation between pollen viability and fertility rate and emphasized that an increase in temperature reduced PV and SF. The correlation graph indicated that traits other than PV and SF may determine heat tolerance in rice. In the case of the H3 group, there was high pollen viability; however, the SF was comparable to that of the H2 and H4 groups. The low SF in H3 could have been due to the inability of the pollen to germinate or due to the lower number of pollen (Prasad et al., 2006). Thus, QTL other than *qHTSF1.1* and *qHTSF4.1* may also exist that could be present in the donor N22. Other QTL for heat tolerance were reported on chromosomes 5, 7, 9 and 11 that were associated to spikelet fertility (Ye et al., 2012). It is likely that N22 alleles at the *qHTST1.1* and *qHTST4.1* loci interact with those QTL to confer heat tolerance to the well-known N22 heat tolerant variety. Therefore, marker-assisted backcrossing (MABC) to confer heat tolerance in rice could be limited to the introgression of N22 only at *qHTST4.1,* thus suppressing the negative interaction with the N22 allele at *qHTST1.1* that prevents SF stability under heat stress. The fact that HXBF2 and GCP, H2 and H4 had unexpectedly high SF values, considering their reduced PV scores, also suggested that other traits could be useful parameters in determining tolerance to heat stress. Among such other traits, pollen germination and tube growth are known to be affected by heat stress and could also be traits to examine to understand the relationship between heat tolerance and spikelet fertility (Onoriode et al., 2015; Coast et al., 2016; Shi et al., 2017, 2018; Zhang et al., 2018). The current study enabled the identification of a molecular marker for heat tolerance that could be used in rice breeding programs. Furthermore, it seems very timely that heat tolerance should be included in improving rice to effectively thrive under the current climate change scenario.

Conclusion

Four new markers were used to follow heat tolerance QTL to select rice genotypes carrying the target allele on *qHTST1.1* and *qHTST4.1*. The efficiency of marker-assisted selection was confirmed in the F_2 3-way population phenotype for pollen viability and spikelet fertility under heat stress conditions. Lines carrying the N22 allele at *qHTST4.1* had the highest percentage of viable pollen and fertile spikelets under heat stress. Notably, understanding the interaction among the N22 alleles at the two targeted QTL and identifying the other loci contributed by N22 would explain why N22 has superior heat tolerance than the introgression lines carrying the N22 alleles at the two QTL. Nonetheless, the gain realized in heat tolerance with the introgression of the N22 allele at *qHTST4.1* should be further tested, in a more advanced backcrossed generation, to verify the stability of spikelet fertility in the rice exposed to high day temperatures.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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