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ORIGINAL PAPER

Fine-mapping and validating *qHTSF4.1* to increase spikelet fertility under heat stress at flowering in rice

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Abstract

Key message **This study fine mapped and validated a QTL on rice chromosome 4 that increases spikelet fertility under high temperature (over 37 °C) at the flowering stage.**

Abstract Climate change has a negative effect on crop production and food security. Understanding the genetic mechanism of heat tolerance and developing heat-tolerant varieties is essential to cope with future global warming. Previously, we reported on a QTL (*qHTSF4.1*) from an IR64/N22 population responsible for rice spikelet fertility under high-temperature stress at the flowering stage. To further fine map and validate the effect of *qHTSF4.1*, PCR-based SNP markers were developed and used to genotype BC_2F_2, BC_3F_2, BC_3F_3, and BC_5F_2 populations from the same cross. The interval of the QTL was narrowed down to about 1.2 Mb; however, further recombination was not identified even with a large BC_5F_2 population that was subsequently developed and screened. The sequence in the QTL region is highly conserved and a large number of genes in the same gene family were observed to be clustered in the region. The QTL *qHTSF4.1* consistently increased spikelet fertility in all of the backcross populations. This was confirmed using 24 rice varieties. Most of the rice varieties with the QTL showed a certain degree of heat tolerance under high-temperature conditions. In a BC_5F_2 population with clean background of IR64, QTL *qHTSF4.1* increased spikelet fertility by about 15 %. It could be an important source for enhancing heat tolerance in rice at the flowering stage. PCR-based SNP markers developed in this study can be used for QTL introgression and for pyramiding with other agronomically important QTLs/genes through marker-assisted selection.

Abbreviations

MAGIC	Multiparent advanced generation intercross
PCR	Polymerase chain reaction
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphism

Introduction

Climate change has become a serious concern since the late 20th century. Recent global warming has resulted in more frequent extreme weather episodes such as heat and cold. The frequency of heat wave occurrence has increased in large parts of Europe, Asia and Australia since 1950 (IPCC 2013). These changes have significant negative effects on crop production and global food security, which will be greatly challenged under future projected global warming scenarios.

As the staple food for more than half of the world population, rice is widely grown in many tropical and temperate countries. Rice yield losses due to high temperature have been reported in many countries across continents, such as Pakistan, India, Bangladesh, China, Thailand, Japan,

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Australia and the US (Hasegawa et al. 2009; Matsushima et al. 1982; Osada et al. 1973; Tian et al. 2009). Significant yield losses have also been predicted because of future global warming (Basak et al. 2010; Battisti and Naylor 2009; Cline 2008; Karim et al. 2012; Lobell et al. 2008). Hence, introgressing heat tolerance into rice and other major crops should be considered a high priority when developing new varieties for regions frequently threatened by heat waves.

Early studies showed that rice is very susceptible to high temperature, especially at the reproductive and grain-filling stages (Satake and Yoshida 1978; Sato et al. 1973). High temperature of over 35 °C at the flowering stage reduces pollen viability and increases spikelet sterility, which leads to significant yield losses, low grain quality, and low harvest index (Matsui et al. 1997a, b; Matsushima et al. 1982; Osada et al. 1973; Prasad et al. 2006; Zhong et al. 2005). Fortunately, rice varieties tolerant of high temperature have been reported in different studies (Matsui et al. 1997a, 2001; Tenorio et al. 2013). Breeding populations have been developed using these heat-tolerant varieties, and more than 30 QTLs associated with heat tolerance have been identified in different populations (Cao et al. 2003; Chen et al. 2008; Cheng et al. 2012; Jagadish et al. 2010; Xiao et al. 2011; Ye et al. 2012, 2015; Zhang et al. 2008, 2009). However, none of these QTLs has been fine mapped or validated. Since it is difficult to precisely evaluate heat tolerance across breeding programs because of temperature variation in the field, characterizing identified QTLs and developing markers for selection at earlier generations without the need for high-temperature treatment will be more efficient in improving heat tolerance in new varieties.

We have developed a precise method for evaluating rice heat tolerance and identified two QTLs controlling spikelet fertility under high-temperature conditions using an IR64/N22 population (Ye et al. 2012). The QTL on chromosome 4 (*qHTSF4.1*) was consistently identified in different populations (Xiao et al. 2011; Ye et al. 2012, 2015). This QTL is a promising candidate for improving heat tolerance in new rice varieties. This study was designed to fine-map and validate *qHTSF4.1* using several backcross populations.

Materials and methods

Development of mapping populations

Heat tolerance QTL *qHTSF4.1* was previously mapped on chromosome 4 using an F_2 population derived from the cross IR64/N22 (Ye et al. 2012). At the same time, selected F_2 plants with *qHTSF4.1* were backcrossed with IR64 to develop a BC_2F_2 population for fine mapping. The selected BC_2F_2 plants with this QTL were further backcrossed to IR64 to develop a BC_3F_2 population for further fine mapping. Selected plants with *qHTSF4.1* were continuously backcrossed with IR64 to produce a BC_5F_2 population for evaluating the contribution of this QTL in pure background of IR64 (Supplemental Figure 1). To validate the effect of the QTL in different genetic backgrounds, 24 rice varieties/breeding lines, including 10 *indica* MAGIC lines (Bandillo et al. 2013), were genotyped using SNP markers in the QTL region, and evaluated for heat tolerance using temperature-controlled growth chambers.

Phenotyping for heat tolerance

Seeds of IR64, N22, and the backcross progenies were germinated and sown in plastic trays, and 21-day-old seedlings were transplanted into small plastic pots (10 cm in diameter) filled with natural clay loam soil, one plant per pot. The pots were randomly arranged in a net-house and the plants were grown under natural temperature and sunlight till heading. When each plant started heading, 3–5 uniform panicles were marked and the plant was moved into an indoor growth chamber (IGC, Thermoline, Australia). The date of high-temperature treatment commencement (heading date) was recorded. The temperature set in the IGC was the same as described by Ye et al. (2012), with 6 h of high temperature (38 °C) each day during flowering time (0830–1430 hours) (Supplemental Table 1). After 14-day of exposure to high temperature, the plants were moved back to the net-house and grown to maturity. At physiological maturity, plant height (measured from soil surface to the panicle tip), panicle neck length (exsertion, from flag leaf collar to panicle node), panicle length, flag leaf length, number of fully filled spikelets (including partially filled) and empty spikelets were recorded. The mean spikelet fertility of three uniform panicles was used to evaluate the heat tolerance of the plant.

Development of PCR-based SNP markers

Based on QTL mapping results from the IR64/N22 population (Ye et al. 2012), potential polymorphic SNPs in the QTL region were identified by searching the OryzaSNP database (http://oryzasnp.plantbiology.msu.edu/index.html). The DNA sequences flanking the SNPs were downloaded from the database, and Tetra-primer ARMS PCR primers (Ye et al. 2001) were designed using the online tool BatchPrimer3 (http://batchprimer3.bioinformatics.ucdavis.edu/index.html) (You et al. 2008). In total, 220 primers were designed for 55 SNPs, and 13 SNP markers (52 primers) evenly distributed in the QTL region were used for fine-mapping and marker-assisted selection (MAS) in this study (Table 1).

Table 1 List of SNP markers used for marker-assisted selection

Marker	SNP position (bp)	Primer	Sequence	Product size (bp)
M4	17,028,092	Outer forward	CATGTGTTTTCTTGTAGAACTTTAG	186/214
		Outer reverse	TCAAGACTATTTTATCAATCGTTAC	
		Inner forward	CTCTACGTTTCAACTGAGCA	
		Inner reverse	AAGAGATCGCATGTGGAC	
M49	17,236,304	Outer forward	GTTGAAGTCCATCCTTTTCT	191/157
		Outer reverse	TCATTTCCCACATTCATATT	
		Inner forward	GTGGCTCAGGTTGCAT	
		Inner reverse	CTAGGAGAGCGGACCTG	
M85	17,483,706	Outer forward	TGCTTAAAAGATCATCACCC	279/231
		Outer reverse	CTCACCATAGCAACTTCAGG	
		Inner forward	GTCCATGTCATGTTTCGACT	
		Inner reverse	CTATGTCATACGAATGCTACAAGAT	
M86	17,685,476	Outer forward	AAGTTAAAATGTCAGCTCGG	292/247
		Outer reverse	AATAATTGATGGCTTCGAGA	
		Inner forward	GCCTATCCAACAAGGGAATA	
		Inner reverse	ATTGAAAAGAATTCTAGCACCAC	
M87	17,838,838	Outer forward	TGATCGGACTTTTCAGTTTT	239/193
		Outer reverse	ACAAGTGCAGCAAGTTTGTA	
		Inner forward	CCCCAGCTAGTGTACTGAATTATC	
		Inner reverse	CCAACACCAAGTTTGACTCC	
M13/M51	17,877,584	Outer forward	GGATGATTTGTGAATCTTCTATTCT	224/196
		Outer reverse	CCTTTGAAAAATTATTTGTTGAGTA	
		Inner forward	GTAGGAAACGAGAGATCAATCC	
		Inner reverse	AAGCATCACACGGTTGTACTTA	
M73	18,010,335	Outer forward	TGCAGTTTATAAGCGGAATTT	183/164
		Outer reverse	GTGACATGGCGTCCGTA	
		Inner forward	AACTGCAAAGCTGAATGGATA	
		Inner reverse	CCAAGGTACACTATACTTGCATTTC	
M77	18,480,809	Outer forward	TGTATCAAGCCATCATTTGG	193/249
		Outer reverse	ATGGCCTAGTTGGACATCAT	
		Inner forward	ATGTGAGACTATGGAACGCA	
		Inner reverse	GACTATCATCTTGAATTTCTTCGTC	
M80	18,555,670	Outer forward	TTTGACTAAATTTGGTGACAGGTAT	356/270
		Outer reverse	ATGATCAGAAAAAGAAAAGGAACTT	
		Inner forward	TTCATTTCCATGATCCAGAAG	
		Inner reverse	CACCCTACAAAGTGCAATGG	
M81	18,587,987	Outer forward	TCCATGTGTATGTTGTCTCTCA	171/243
		Outer reverse	AAAGACCTTTGCGTTTGTCT	
		Inner forward	CCATCCTTTATTTGGAAAGCTA	
		Inner reverse	TGGGATTTAGTAACGTTTTCTTTC	
M83	18,716,672	Outer forward	TAGCTGTGTGCTCGTGATTT	291/263
		Outer reverse	GGATGTACGGTTGAGGATTT	
		Inner forward	GTAAGCCAATTTTGACGCA	
		Inner reverse	CTCCCGTACCTTTCTTACCC	
M84	18,992,058	Outer forward	ACTGCTCATCAAATATGCCT	274/215
		Outer reverse	TGTGAGATGTAAAAACCAACC	
		Inner forward	AACTTCTATCTGAAATGCTTATGG	
		Inner reverse	CCAAGGCTAACAAGTCGTAG	

Table 1 continued

Marker	SNP position (bp)	Primer	Sequence	Product size (bp)
M24	19,381,891	Outer forward	TGAAACTTTAACAATAATTGAGGAG	227/194
		Outer reverse	AAGAGTATTCAAGCTCTCTCTCTCT	
		Inner forward	AAATGCATACAGAATATGGAATTTA	
		Inner reverse	TACATATCCTAATCAAAGCATGTTC	

Genotyping using PCR-based SNP markers

Twenty-one days after seeding, a young leaf (about 10 cm long) from each plant was collected and frozen for DNA extraction. Genomic DNA of IR64, N22, and their progeny was extracted using SDS extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl, 1.25 % SDS and 1 % v/v 2-mercaptoethanol) and chloroform/isoamylalcohol (24:1) solution, followed by ethanol precipitation. The RNA was digested by RNase at 37 °C for 30 min. The final concentration of the DNA samples was normalized to 10 ng/μl for genotyping using PCR-based SNP markers.

The PCR for SNP markers was carried out using QIAGEN Multiplex PCR kit (QIAGEN, Venlo, Limburg, Netherlands). The PCR cocktail contains 4 μl 2 × Multiplex PCR master mix, 1 μl of outer primers mix (1 μM each), 1 μl of inner primers mix (10 μM each), 1 μl of DNA (10 ng/μl), and 1 μl of distilled water. The PCR program was set at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 57 °C for 90 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were run on 8 % (w/v) polyacrylamide gel for size separation using an MGV-202-33 vertical gel electrophoresis system (CBS Scientific Co.). The gel was then soaked in SYBR® Safe DNA gel-staining solution and visualized with a UV transilluminator. The DNA band of IR64 genotype was coded as AA, N22 genotype as BB, and heterozygote as AB.

To check the background of the selected BC_2F_2 plants, the genome DNA of those plants were genotyped using Illumina BeadXpress 384-plex SNP plates GS0011861 (customized for Indica–Indica). The custom oligo pool assay (OPA), which contained 384 well-distributed SNPs per assay, was designed by Cornell University (Thomson et al. 2012; Zhao et al. 2010) from a high-quality subset of the SNPs discovered in 20 diverse *O. sativa* landraces (McNally et al. 2009). The background of selected BC_5F_2 plant was checked using Illumina Infinium 6 K SNP beadchip. The 6 K SNP beadchip was designed for high-resolution genotyping by Dr Susan MacCouch's group at Cornell University. PCR amplification and hybridization were carried out following the user manual (Illumina, San Diego, CA).

Statistical analysis

The mean spikelet fertility of different genotypes was compared by one-way ANOVA using MINITAB V14.0 (Minitab Inc.). The SNP data for background selection were analyzed using GGT2.0 (Ralph 2008). In the GGT data file, the IR64 genotype was coded as A, N22 genotype as B, and the heterozygote as H.

Results

Fine mapping using the BC_2F_2 population

Two SNP markers (M13 and M24) flanking the QTL region were used for genotyping 660 BC_2F_2 plants. Among them, 18 plants showed recombination between M13 and M24. Another nine new SNP markers were used to genotype the 18 selected BC_2F_2 plants. The chromosomal crossovers of all the plants are located between SNP markers M83 and M84 (Fig. 1).

Based on the genotyping data, the 18 selected and 111 other BC_2F_2 plants that were randomly picked, along with their parents, IR64 and N22 (10 plants each) were planted in the greenhouse and treated in the growth chambers at the flowering stage. A significant difference in spikelet fertility was observed among the genotypes of SNP marker M83 in both selected and random BC_2F_2 plants, whereas no difference was observed for marker M84 (Fig. 2). Thus, the QTL *qHTSF4.1* is more tightly linked to M83 than to M84. A heat-tolerant plant with N22 genotype at M83 and IR64 genotype at M84 was selected for backcrossing and developing a BC3F2 population for further fine mapping.

The background of selected BC_2F_2 plants were checked using the same set of 384-plex SNP markers that were used for QTL mapping. In the BC_2F_2 plants, some large chromosomal introgressions from N22 were still present (supplemental Figure 2).

Fine mapping using a BC_3F_2 population

Two SNP markers, M41 and M77, were used for genotyping 1920 BC_3F_2 plants. Among them, only one plant showed recombination between the two markers. By genotyping

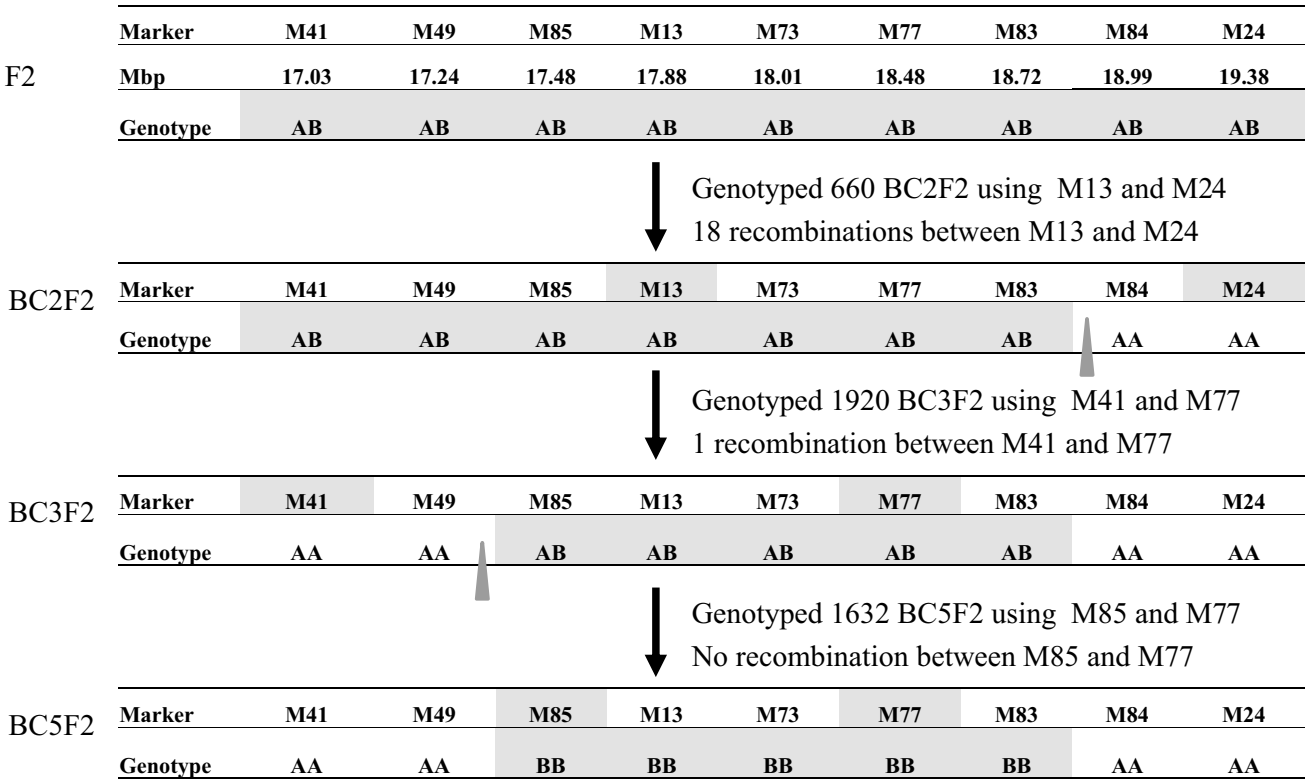

Fig. 1 Fine mapping and marker-assisted selection of *qHTSF4.1* using BC_2F_2, BC_3F_2, and BC_5F_2 populations. Two flanking markers were used for genotyping the populatrions, and the other markers were used for genotype the selected plants with recombination between the flanking markers. Mbp indicate the physical position of the SNP in million base pairs. AA is IR64 genotype, AB is heterozygote, BB is N22 genotype. The triangles show the positions of recombination

more SNP markers between M41 and M77, the chromosomal crossover was found between SNP markers M49 and M85 (Fig. 1). This plant was heterozygous between markers M85 and M83, thus, it was self-pollinated to produce a BC_3F_3 population to confirm the effect of the QTL. At the same time, this BC_3F_2 plant was continuously backcrossed with IR64 to produce BC_4F_1 and BC_5F_2 populations. The distance between M85 and M83 is about 1.2 Mb.

Genetic effect of *qHTSF4.1* in BC_3F_3 population

A total of 768 BC_3F_3 plants were genotyped using SNP markers M85 and M73. Then, 50 plants each with the genotype AA, AB, and BB, along with 50 plants each of the parents, IR64 and N22, were phenotyped for heat tolerance (15 plants × 3 temperature treatments, plus 5 plants as control). At the flowering stage, fifteen plants of each genotype were treated at 37, 38, and 39 °C for 14 days, whereas the remaining five plants were maintained in the greenhouse as control. The results showed that the plants with *qHTSF4.1* (BB) are consistently more tolerant of high-temperature stress at flowering stage in all the three temperature treatments, especially at 38 and 39 °C. The QTL introgressed plants maintained relatively higher spikelet fertility (>32.7 %), while the other genotypes recorded very low spikelet fertility (<22.5 %) (Fig. 3).

Validating the effect of *qHTSF4.1* in BC_5F_2 population

To identify recombinants in the QTL region and further narrow down the QTL interval, 1632 BC_5F_2 plants were genotyped using SNP markers M85 and M77. However, no recombination was found between the two markers. Based on marker genotyping, 20 plants of each genotype (AA, AB, and BB), along with 20 plants of IR64, were phenotyped for heat tolerance at the flowering stage. The results also clearly showed that the spikelet fertility of plants with *qHTSF4.1* (BB = 44.6 ± 13.1 %) were significantly higher ($F = 12.5$, $p < 0.001$) than other genotypes (AA = 27.1 ± 9.6 %, AB = 26.7 ± 11.1 %) and IR64 (19.4 ± 8.4 %). In both BC_3F_3 and BC_5F_2 populations, plants with *qHTSF4.1* showed an increase of about 15 % in spikelet fertility when exposed to 38 °C during flowering.

The background of selected BC_5F_2 plants were checked using the 6 K high density SNP markers. In a BC_5F_2 plant, there were only two small fragments from N22 located on

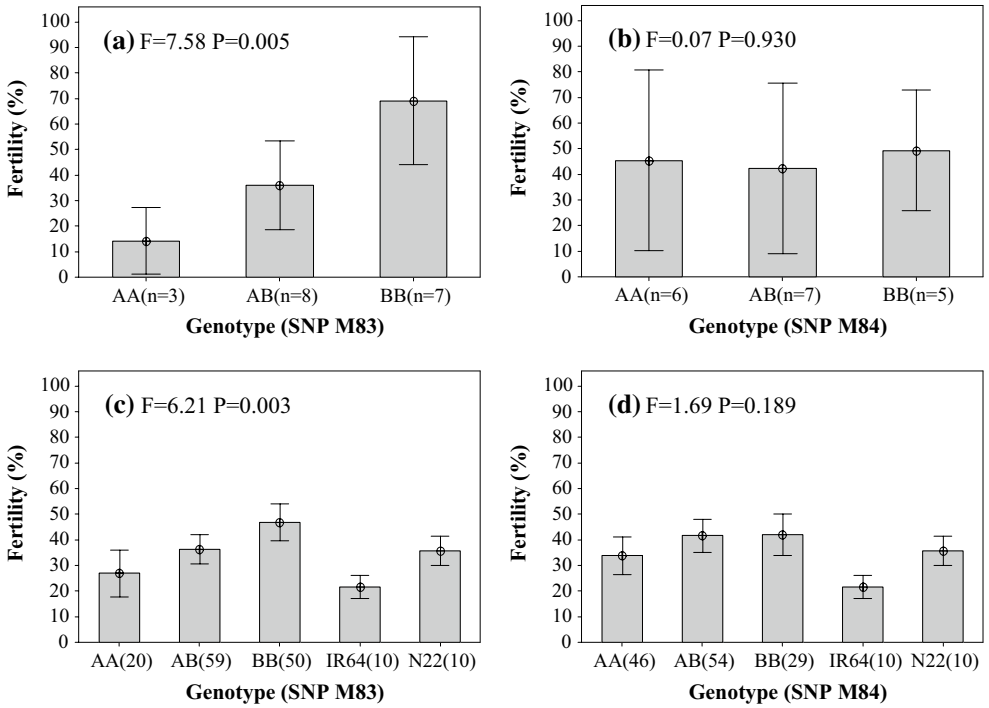

Fig. 2 Spikelet fertility of different genotypes of SNP markers M83 and M84 in (**a**, **b**) selected and (**c**, **d**) random BC_2F_2 populations. In random population, one-way ANOVA was done for BC_2F_2 plants only, and did not include the parents. AA is IR64 genotype, BB is N22 genotype, and AB is the heterozygote

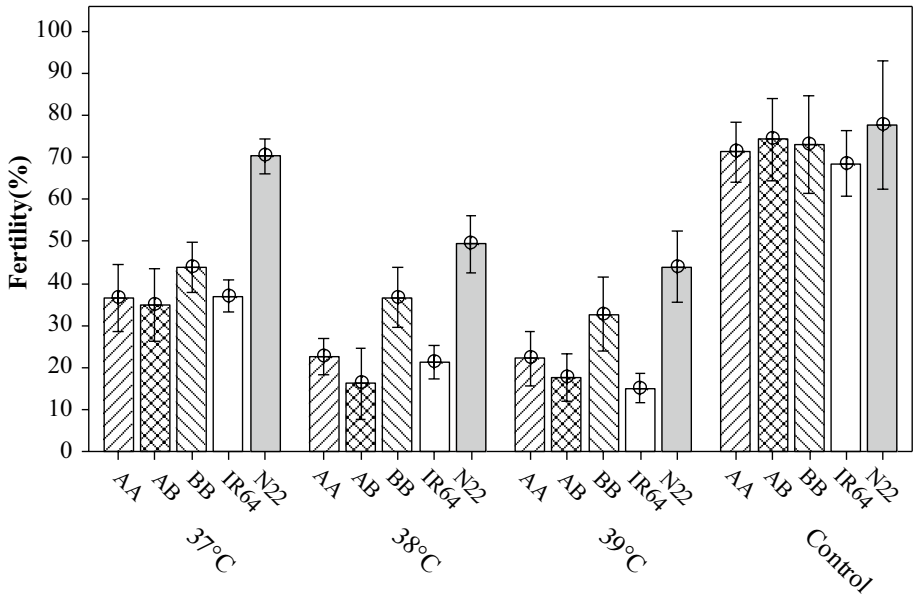

Fig. 3 Spikelet fertility of BC_3F_3 lines under different high-temperature treatments. AA is IR64 genotype, BB is N22 genotype, and AB is heterozygote. The *bars* represent the 95 % confidence interval for mean

chromosomes 4 and 5 (Supplemental Figure 2). Including the fragment on chromosome 4 (target QTL locus), more than 99 % of the genome (4.1 Mb out of 430 Mb) is already same as IR64. The morphological traits of different backcross populations also showed that, after 3 cycles of backcrossing, the progenies were already similar to the recurrent parent IR64 (Table 2).

Validating the effect of *qHTSF4.1* in rice varieties

To validate the effect of *qHTSF4.1* in natural populations, 24 rice varieties/breeding lines, including 10 *indica* MAGIC lines, were genotyped using the SNP markers in the QTL region and phenotyped for heat tolerance at the flowering stage. Three MAGIC lines (4, 11, and 74)

Table 2 Comparison of agronomic characters between backcross lines and their parents

Traits	Genotype	BC_2F_2	BC_3F_3	BC_5F_2
Days to heading	AA	72.9 ± 3.1	67.8 ± 1.3	65.2 ± 1.4
	AB	74.1 ± 2.6	67.7 ± 1.2	64.4 ± 1.5
	BB	73.6 ± 2.7	67.9 ± 1.0	64.9 ± 1.4
	IR64	75.7 ± 1.3	68.1 ± 1.1	64.7 ± 1.2
	N22	64.0 ± 1.0	63.6 ± 1.1	–
Plant height (cm)	AA	88.2 ± 8.7	105.2 ± 7.2	101.3 ± 4.3
	AB	88.0 ± 8.0	105.4 ± 9.1	101.3 ± 6.6
	BB	86.0 ± 14.8	107.5 ± 6.8	101.2 ± 4.7
	IR64	98.7 ± 3.0	121.3 ± 4.7	103.3 ± 3.6
	N22	124.3 ± 1.5	151.7 ± 14.0	–
Panicle length (cm)	AA	26.1 ± 1.7	24.7 ± 2.6	27.4 ± 1.6
	AB	25.9 ± 1.8	24.8 ± 2.2	27.4 ± 2.5
	BB	25.7 ± 2.0	25.5 ± 2.4	27.6 ± 1.1
	IR64	25.5 ± 2.0	24.3 ± 2.1	27.3 ± 1.9
	N22	22.4 ± 1.5	23.3 ± 4.0	–
Flag leaf length (cm)	AA	35.2 ± 4.8	32.6 ± 5.1	41.2 ± 8.7
	AB	34.5 ± 5.7	32.5 ± 5.5	44.1 ± 6.6
	BB	34.8 ± 5.5	34.6 ± 7.2	40.4 ± 9.5
	IR64	35.9 ± 6.8	31.7 ± 6.2	40.2 ± 6.0
	N22	35.2 ± 6.4	47.7 ± 7.6	–
Total spikelet per panicle	AA	112.2 ± 19.5	105.9 ± 17.0	124.2 ± 14.9
	AB	114.0 ± 23.6	104.2 ± 14.7	123.1 ± 16.4
	BB	112.6 ± 23.9	109.8 ± 16.6	123.6 ± 17.5
	IR64	117.1 ± 11.4	106.2 ± 20.0	125.9 ± 17.2
	N22	129.0 ± 2.7	113.9 ± 15.1	–

Data were shown as mean ± standard deviation

AA IR64 genotype, *BB* N22 genotype, *AB* heterozygote

and Dasan showed different haplotypes, but there was no recombination in the QTL region for the other varieties. This confirmed that the QTL region is conserved. These SNP markers can well distinguish the heat tolerance of most of the genotypes, except for five of them (MAGIC47, MAGIC56, MAGIC74, IR2307, and Dasan). The heat tolerance of those five lines is possibly affected by other QTLs. In general, the AA genotype is more sensitive to heat stress than the BB genotype (Table 3).

Discussion

Fine mapping of *qHTSF4.1*

Plant responses to heat stress can potentially be affected by environmental factors, especially by developmental stage, temperature, and relative humidity. Stability of the environment is the key determining factor in obtaining reliable and repeatable phenotyping results. By precisely controlling temperature and humidity in the growth chambers, we were able to detect and fine map the QTL (*qHTSF4.1*) identified in an earlier study (Ye et al. 2012). In the BC_2F_2 population, we were able to identify 18 plants with recombination in the QTL region, and anchor the right flanking marker M83. In the BC_3F_2 population, only one plants showed recombination in the QTL region. By genotyping and phenotyping the following BC_3F_3 population, the results confirmed that *qHTSF4.1* is located between marker M85 and M83. The physical distance between marker M85 and M83 is about 1.2 Mb. To further narrow down the QTL interval, 1632 BC_5F_2 plants were genotyped using SNP markers M85 and M77. But no recombination was found between the two markers. Thus, the recombination rate in the QTL region is very low, map-based cloning strategy is not efficient. By continuous marker selection and backcrossing, the background of the BC_5F_2 plant is already highly similar to IR64 (>99 %), and we already got a BC_6F_2 plant with only the 1.2 Mb introgression of *qHTSF4.1* from N22. It is possible to use it for transcriptional analysis (RNA sequencing) to identify potential candidate genes (Zhang et al. 2010).

We screened a large number of BC_2F_2, BC_3F_2, and BC_5F_2 plants using the markers in the QTL region to narrow down the QTL interval. However, very few recombinant plants were identified. The results indicated that the QTL is located in a highly conserved region, with genes tightly linked and inherited together. This is generally seen with QTLs closer to the centromere region, although *qHTSF4.1* is not very close to the centromere (about 8 Mb away). Wu et al. (2003) indicated that the ratio of physical-to-genetic distance in the centromere regions was up to 2740 kb per cm, or 10 times higher than that throughout the rest of the genome (250–300 kb/cm). The low recombination rate may be due to abundance of repetitive sequences near the centromere and transposon/retrotransposon-rich regions. The low recombination within the QTL region limited mapping resolution and discouraged further efforts for screening of recombinant with markers. Hence, additional advanced crossing approaches, such as the multiparent advanced generation intercross (MAGIC), are needed to break this linkage.

Within the 1.2 Mb QTL interval, there are about 200 genes (see Supplemental Table 2). However, 53 of the genes are transposon and retrotransposon proteins, 49 genes are hypothetical proteins with known or unknown functions, and 16 genes are expressed protein with unclassified function. Many of the genes belong to the same gene families. For example, there are 24 cell wall-associated receptor kinase (WAK) genes in a 540-kb region (17.41–17.95 Mb) and six rapid alkalinization factor (RALF) genes in an 80-kb region (18.61–18.69 Mb). This indicates that the sequence in the QTL region is highly duplicated. The expression of a trait could result from the contribution of

Table 3 Genotypic and phenotypic variation among 24 rice varieties

Variety	Sub-group	M85	M86	M87	M51	M80	M81	M83	M84	Fertility (%)	Heat tolerance
IR28	Ind	AA	AA	AA	AA	AA	AA	AA	AA	5.3	S
MAGIC11	Ind	AA	AA	AA	AA	AA	BB	BB	BB	5.7	S
PsBRc94	Ind	AA	AA	AA	AA	AA	AA	AA	AA	8.3	S
IR72	Ind	AA	AA	AA	AA	AA	AA	AA	AA	9.0	S
IR64	Ind	AA	AA	AA	AA	AA	AA	AA	AA	9.0	S
MAGIC4	Ind	AA	AA	AA	AA	AA	AA	AA	BB	20.8	M
MAGIC16	Ind	AA	AA	AA	AA	AA	AA	AA	AA	24.4	M
MAGIC47	Ind	AA	AA	AA	AA	AA	AA	AA	AA	45.8	T
MAGIC56	Ind	AA	AA	AA	AA	AA	AA	AA	AA	49.6	T
IR2307	Ind	AA	AA	AA	AA	AA	AA	AA	AA	59.9	T
MAGIC74	Ind	AA	BB	BB	AA	AA	AA	AA	AA	53.5	T
Dasan	Jap	BB	BB	BB	BB	BB	BB	BB	AA	15.1	S
Keunseom	Jap	BB	BB	BB	BB	BB	BB	BB	BB	22.8	M
Todorokiwase	Jap	BB	BB	BB	BB	BB	BB	BB	BB	27.8	M
TR22183	Jap	BB	BB	BB	BB	BB	BB	BB	BB	30.2	M
Milyang23	I/J	BB	BB	BB	BB	BB	BB	BB	BB	36.7	T
MAGIC122	Ind	BB	BB	BB	BB	BB	BB	BB	BB	38.0	T
MAGIC79	Ind	BB	BB	BB	BB	BB	BB	BB	BB	39.5	T
Chengcheong	Jap	BB	BB	BB	BB	BB	BB	BB	BB	40.9	T
N22	Aus	BB	BB	BB	BB	BB	BB	BB	BB	41.7	T
MAGIC66	Ind	BB	BB	BB	BB	BB	BB	BB	BB	42.4	T
Giza178	I/J	BB	BB	BB	BB	BB	BB	BB	BB	43.8	T
MAGIC45	Ind	BB	BB	BB	BB	BB	BB	BB	BB	50.9	T
IR2006	Ind	BB	BB	BB	BB	BB	BB	BB	BB	58.6	T

Genotype AA is IR64 genotype, and BB is N22 genotype. Sub-group Ind is Indica, Jap is Japonica, I/J is bred from Indica and Japonica cross. Fisher's LSD for spikelet fertility is 14.9

many genes with similar or complementary functions. The heat-tolerant MAGIC74 has a small segment similar to the N22 genotype in the QTL region. It is yet to be confirmed if this segment in MAGIC74 is genuinely associated with heat tolerance, but many cell wall-associated kinase (WAK) genes are located in this small segment. WAK genes are receptor-like kinases that have been implicated in cell wall expansion during development (Sharma et al. 2011, 2013). Recent studies demonstrated that WAKs and WAK-like (WAKL) genes play important roles in cell expansion and tolerance of biotic and abiotic stresses (Brutus et al. 2010; He et al. 1998; Kohorn and Kohorn 2012; Kohorn et al. 2012; Zhang et al. 2005) such as rice blast (Li et al. 2009), bacteria leaf blight (Narsai et al. 2013; Seo et al. 2011), and chilling (Yan et al. 2006). Further studies using isogenic lines are needed to determine the candidate genes involved in heat tolerance.

Validating *qHTSF4.1*

In both a small BC_2F_2 population and a larger BC_3F_3 population, the average spikelet fertility of the plants with *qHTSF4.1* was significantly higher than those without the QTL, as well as than the recipient variety IR64. In the BC_5F_2 population, the background of the genome was almost the same as IR64, the plants introgressed with *qHTSF4.1* clearly showing about 15 % higher spikelet fertility than those without the QTL. This is an important advancement toward maintaining yield stability with the increase in heat stress incidence at the sensitive flowering stage.

Even though the 24 varieties evaluated in this study have very different background, and there are many other QTLs in those varieties; however, the trend of heat tolerance predicted by *qHTSF4.1* is quite strong, except for a few varieties. Most of the varieties with *qHTSF4.1* showed better heat tolerance than those without. The haplotypes of 24 rice varieties evaluated in this study also showed no recombination in the QTL region in most of these varieties. However, three MAGIC lines (4, 11, and 74) and Dasan showed small variation in the QTL region, indicating that the MAGIC population has a higher recombination rate and could be an ideal option for high-resolution QTL mapping. The heat-tolerant line MAGIC74 has a small segment showing the same

genotype as N22 between markers M86 and M87, with a possibility that the effective QTL located in the small region. However, this needs to be further confirmed by (1) introducing the segment into an IR64 background and evaluating its genetic effect and (2) sequencing to identify possible variation in the chromosomal region. We have also developed an eight-way MAGIC population that includes IR64 and N22 as founder parents. This MAGIC population may provide a higher resolution for fine mapping of *qHTSF4.1* once genotyping and phenotyping works are done.

Marker-assisted selection for *qHTSF4.1*

In both BC_3F_3 and BC_5F_2 populations, the spikelet fertility of AA and AB genotypes were not significantly different, though AB genotype was slightly higher than AA genotype in the small BC_2F_2 population. We also observed some BC_nF_1 plants during the backcross process, the BC_nF_1 plants did not show significant tolerance. This confirmed that *qHTSF4.1* is controlled by a recessive gene, which is consistent with the QTL mapping results from the F_2 population (Ye et al. 2012). Thus, we used BC_2F_2, BC_3F_2, and BC_5F_2 for fine mapping of *qHTSF4.1*. It is more time consuming than fine mapping of dominant genes. Once QTL-linked markers are developed, marker-assisted selection for heat tolerance will be more efficient in breeding programs.

The heat-tolerance QTL on chromosome 4 was identified in different populations of heat-tolerant rice varieties 996, N22, and Giza178 (Xiao et al. 2011; Ye et al. 2012, 2015). A similar haplotype was also found in Milyang23 (Ye et al. 2015) and in 13 out of the 24 rice varieties used in this study. Results from these independent studies confirmed that *qHTSF4.1* could effectively increase spikelet fertility under high-temperature stress at the flowering stage. In the advanced backcross populations (BC_3F_3 and BC_5F_2), selected plants are already very similar to the recurrent parent IR64, and no disadvantages in agronomic parameters were observed. Thus, it appears that linkage drag may not to be a problem for marker selection for this QTL. Since the sequence in the QTL region is very conserved, even though the QTL interval is still relatively large (1.2 Mb), the markers in the QTL region can readily be used for marker-assisted selection (MAS) in other populations and breeding lines. Breeders can pick any marker from the left side (M85, M86, and M87) and right side (M81, M83, and M84) for MAS.

QTLs for rice heat tolerance at flowering have been mapped on all chromosomes using various rice populations (Cao et al. 2003; Chen et al. 2008; Cheng et al. 2012; Jagadish et al. 2010; Xiao et al. 2011; Zhang et al. 2009, 2008). However, the additive effect of each QTL is low. Introducing one or a few QTLs into a variety may not sufficiently increase its heat tolerance. Therefore, it is necessary to validate and characterize more QTLs and design functional SNP chips with QTL-linked markers to accelerate selection and incorporation of multiple QTLs and to improve the efficiency of heat-tolerance breeding.

Author contribution statement CY, EDR, KSVJ, and GG designed the experiments. CY and FAT conducted the experiments. CY, PSRM, and GAC analyzed the data and wrote the paper. All authors read and approved the final version.

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

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