

Quantitative Trait Loci Associated with Pollen Fertility under High Temperature Stress at Flowering Stage in Rice (*Oryza sativa*)

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Abstract: High temperature stress (HTS), an increasingly important problem in rice production, significantly reduces rice yield by reducing pollen fertility (PF) and seed setting percentage (SSP). Breeding rice varieties with tolerance to HTS at the flowering stage is therefore essential for maintaining rice production as the climate continues to become warm. In this study, two quantitative trait loci (QTLs) underlying tolerance to HTS were identified using the recombinant inbred lines (RILs) derived from a cross between the HTS-tolerant rice cultivar 996 and the sensitive cultivar 4628. PF was used as the heat-tolerance indicator for the lines subjected to HTS at the flowering stage in field experiments. Two QTLs that affected PF were detected in the interval between RM5687 and RM471 on chromosome 4, and between RM190 and RM225 on chromosome 6, respectively, by using composite interval mapping (CIM) analysis. *qPF4*, the QTL located on chromosome 4, explained 15.1% of the total phenotypic variation in PF, and increased the PF of the plants subjected to HTS by 7.15%. *qPF6*, the other QTL located on chromosome 6, explained 9.31% of the total phenotypic variation in PF, and increased the PF of the plants subjected to HTS by 5.25%. The positive additive effects of the two QTL were derived from the 996 alleles. The two major QTL identified in this study should be useful for further fine mapping and cloning of these genes and for molecular marker-aided breeding of heat-tolerant rice cultivars.

Key words: rice (*Oryza sativa* L.); quantitative trait loci; pollen fertility; high temperature stress

Rice often encounters various adverse climates during its growing season. High temperature stress (HTS), which occurred more frequently in southern China where is the major rice planted area, has become one of the most harmful climate factors to rice yield and the stability of rice production (Zou et al, 2009). In recent years, high temperature stress has shown a growing trend, especially short-term extremely high temperature, resulted from the global greenhouse effect. In 2003, high temperature with an average daily temperature above 38°C and daily maximum temperature of 41.3°C lasted for over 20 days from late July to early August when rice was developing reproductive organs and flowering, and had resulted in a severe loss of rice production on a large scale of rice planting areas in China (Zou et al, 2009). Over 800 000 hectares of midseason rice in Hubei and Anhui Provinces suffered losses of more than 1 500 000 tons of grain yield in 2003 (Xia and Qi, 2004; Yang et al, 2004). With the background of the

global climate continues to become warm and the frequent occurrence of short-term extremely high temperature, to improve the stability of rice production by breeding rice varieties with heat tolerance has become one of the critical objectives for rice breeders and agronomists.

Screen for rice germplasm with tolerance to HTS was initiated in Japan and at the International Rice Research Institute (2007) in 1970s. Rice germplasm has been screened for tolerance to HTS by exposing the plants to temperatures as high as 41°C for 2 h at flowering stage or for the whole crop cycle (Satake and Yoshida 1978). Significant differences in tolerance to HTS during flowering have been identified among rice genotypes in both indica and japonica rice cultivars. Similar results were also observed (Xu et al. 1989).

Several quantitative trait loci (QTL) analyses on HTS tolerance with molecular markers have been reported in recent years. Six HTS QTLs with additive effects, as well as eight pairs of QTLs with additive-additive epistatic effects, were detected with

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the double haploid (DH) population developed from a cross between the indica rice IR64 and the japonica rice Azucena (Cao et al, 2002, 2003). Zhu et al (2005) reported three QTL, which were located on chromosome 1, 4, and 7 and explained 8.9, 17.3 and 13.5%, respectively, of the phenotypic variation in HTS tolerance at the grain-filling stage, by using a backcross inbred line (BIL) population derived from the japonica/indica cross of Nipponbare/Kasalath. Three HTS QTLs were identified from the backcross (BC) population developed from a cross between USSR5 (japonica) and Guangjie 9 (indica) that explained 6.4% to 15.8% of the phenotypic variation (Zhao et al, 2006). Additional QTL associated with HTS tolerance during flowering have been recently identified from RIL populations developed from indica/japonica or indica/indica crosses (Zhang et al, 2008; Chen et al, 2008). All the identified QTL associated with HST tolerance except those reported by Chen et al (2008) were identified from indica/japonica crosses, with the seed setting percentage (SSP) or 1000-grain weight as an indicator. It is difficult to eliminate the influences of the hybrid sterility in indica/japonica hybrids and their progenies, thus the accuracy of QTL mapping was decreased.

Previous QTL mapping of heat tolerance were conducted at the different rice growth stages, and the heat tolerance was also identified with different indicators. It is well known that the period from booting to flowering is the most sensitive stage to HTS in rice (Satake and Yoshida 1978). Daily temperatures higher than 30°C or daily maximum temperatures higher than 35°C during the flowering period will result in poor anther dehiscence and lower pollen production, and hence a small number of germinating pollen grains on the stigma and a low rate of fertilization (Satake and Yoshida 1978; Matsushima et al, 1982; Matsui and Omasa 2002; Prasad et al, 2006). It is concluded that pollen in rice is the most vulnerable organs under high temperature conditions. Nevertheless, there was no report about QTLs underlying HTS by using the pollen fertility as an indicator under HTS during the flowering stage in previous studies.

Stable HTS tolerance has been identified in the indica rice cultivar 996, with consecutive years of

field observation and identification of heat tolerance (Luo et al, 2005). A two-line hybrid rice cross Luliangyou 996 with outstanding heat tolerance, was also bred with 996 as a male parent (Chen et al, 2006). In order to further explore new genes underlying heat-tolerance in rice, the QTL mapping was conducted by using an RIL population derived from a cross between the HTS-tolerant rice cultivar 996 and the sensitive cultivar 4628, and pollen fertility of rice plants subjected to HTS at the flowering stage was used as the heat-tolerance indicator.

MATERIALS AND METHODS

Plant materials

The HTS-tolerant rice cultivar 996 and the sensitive cultivar 4628, were used as the parental lines for the development of the RIL mapping population. F₁s of the 996×4628 cross were advanced to the F₂ generation in 2004. Five to ten seeds from each plant in the F₂ to F₈ generation were planted in Changsha and Hainan, China from 2005 to 2008. Individual F₃ to F₉ families were developed from one of the previous generation plants. The final mapping population consisted of 286 F₉ RILs.

Field planting and rice pollen fertility measurement

In 2009, the RILs and the parents were planted at a rice field covered with a bird net at the Hunan Agricultural University, Changsha, China (28°11'N, 113°E). To ensure that all lines headed at the same time and therefore were exposed to the same HTS conditions at flowering stage, seeds of all the RILs and the parents were sown on May 8, 15, 22 or 28 according to the growth durations of each line and the local historical meteorological data. Twenty seeds of each line were sown in one row with a spacing of 15 cm between rows. The seedlings of each line were thinned to five plants per row, with an in-row spacing of 13 cm, at the five-leaf stage. The field was managed according to local rice culturing procedures, and no disease or insect took place during the whole growth period.

Air temperature measured with a copper-constantan thermocouple at canopy height was

recorded every 5 minutes by data loggers (Zeda Instruments Co. LTD, Hangzhou, China). In 2009, the daily average temperature from July 14 to 18 was 31.17, 30.70, 30.49, 31.00, and 32.32°C, respectively; the corresponding daily maximum temperatures during 13:00–15:00 were 43.5, 41.4, 40.8, 42.7, and 42.6°C, and the daily minimum temperature was about 25°C. The panicles headed on July 14 and 15 were marked with a red fiber rope. On the 3rd day of high temperature treatment, all the anthers of five spikelet just before dehiscence were sampled from the upper of the panicle and stored in 1.5 mL centrifuge tube filled with FAA solution (100 mL, containing 90 mL of 70% alcohol solution, 5 mL glacial acetic acid and 5 mL formaldehyde). The tubes were stored at 4°C refrigerator. Pollens from five anthers were mixed and spread on a microscope slide, and photographed in the microscope of Motic BA300 (Motic China Group Co., Ltd) after stained with an I₂-KI solution containing 0.1% (w/v) iodine and 1% (w/v) iodine potassium. Darkly stained and unstained pollens were counted in three separate visual fields for each individual. The average ratio of the number of darkly stained pollens to the total number of pollens in the above three visual field was used to evaluate the pollen fertility for each RIL.

DNA extraction and genotyping with molecular markers

Leaves from each F₉ RIL were sampled, and genomic DNA was extracted according to Saghi Maroof et al (1994). A total of 862 simple sequence repeat (SSR) and 66 ??? (SFP) primer pairs synthesized by Yingjun Biotechnology Co., Ltd (Shanghai, China) and Genscript Biotechnology Co., Ltd (Nanjing, China) were used to survey the polymorphism between the two parents. The markers are evenly distributed on 12 rice chromosomes according to the results of Chen et al (1997), Temnykh et al (2000), McCouch et al (2002), the International Rice Genome Sequencing Project (2005), and Jeremy et al (2008). The SSR marker assay was conducted as described by Wu and Tanksley (1993). PCRs were conducted in a 10 µL reaction mix containing 37.5 ng template DNA, 1×PCR buffer, 0.025 U of Taq DNA polymerase, 0.2 mmol dNTPs,

and 0.8 pmol forward and reverse primers. PCR amplification was performed with an ABI PCR system 2700. The PCR reactions involved an initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C; and then a final extension for 7 min at 72°C. PCR products were separated on 8% polyacrylamide gels (38:2 acrylamide:bisacrylamide).

Data analysis

Genotypic data of 162 polymorphic SSR and SFP markers were used for the linkage map construction and QTL analysis. The molecular linkage map was constructed using Mapmaker/Exp3.0 at the logarithm of odds (LOD) value of 3.0 (Lincoln et al, 1992). The Kosambi function was used to calculate the genetic distance. *QTL cartographer* was used to identify QTL conferring high temperature tolerance on the basis of composite interval mapping (CIM) analysis (Zeng 1994; Wang et al, 2007). The percentage of total phenotypic variation explained by each QTL and their additive effects were estimated by the same software. Tests were performed at a 2 cM-interval and cofactors were selected by forward/backward stepwise regression (*Model 6*) with *QTL Cartographer v 2.5* (Wang et al, 2007). The significant threshold for CIM was determined by 300 permutation tests (Churchill and Doerge 1994). In this study, the threshold value of LOD was 3.67. The phenotypic variation explained by a QTL (r^2) conditioned by the CIM cofactors included in the model was calculated at the most likely QTL position. The additive effect of an allelic substitution at each QTL was also obtained. The LOD peak of each significant QTL was considered as the QTL location on the linkage map.

RESULTS

Distribution of the pollen fertility (PF) in the RIL population

Under high temperature stress, the pollen fertility percentages of cultivar 996 and 4628 were 91.56% and 63.98%, respectively (Fig. 1). The difference of pollen fertility between the two parents, 996 and 4628,

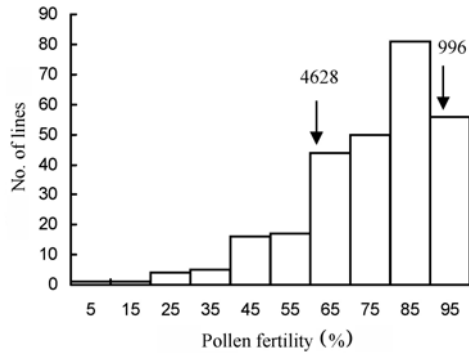


Fig. 1. Frequency distribution of pollen fertility of the recombinant inbred lines grown under high temperature stress at the flowering stage.

was significant at the 0.01 level. A continuous distribution and large variation in pollen fertility were observed in the recombinant inbred lines, with a range of 5.91% to 99.0% (Fig. 1). The pollen fertility also exhibited a skewed population distribution toward higher pollen fertility (Fig. 1). Transgressive segregations were observed in the RIL population, with 41 lines processing the higher pollen fertility percentage than that of the heat-tolerant parent, 996, and 60 lines below the heat-sensitive parent, 4628.

QTL for pollen fertility percentage under HTS

In the total of 928 pairs of SSR and SFP primers, 172 pairs showed polymorphisms between the parental 996 and 4628. Among them, 162 polymorphic SSR and SFP markers distributed on 12 chromosomes were used for the construction of a genetic linkage map using the RIL population. The genetic map contains 15 linkage groups, with the markers of the chromosomes 3, 9 and 11 were distributed in two linkage groups, respectively. The linkage map was 2025.2 cM in length, with an average distance of 12.5 cM between adjacent markers.

Two HTS QTLs, temporarily designated as *qPF4* and *qPF6*, were detected on chromosomes 4 and 6 using the composite interval mapping (CIM) method

Table 1. QTLs identified for pollen fertility under high temperature stress during flowering stage.

QTL	Chr.	Interval	LOD	Add (%) ^a	Var (%) ^b
<i>qPF4</i>	4	RM5687–RM471	7.54	7.15	15.1
<i>qPF6</i>	6	RM190–RM225	4.43	5.25	9.3

^a Additive effect, a positive value indicates the contribution from the 996 allele; ^b Percentage of total phenotypic variance explained by the QTL.

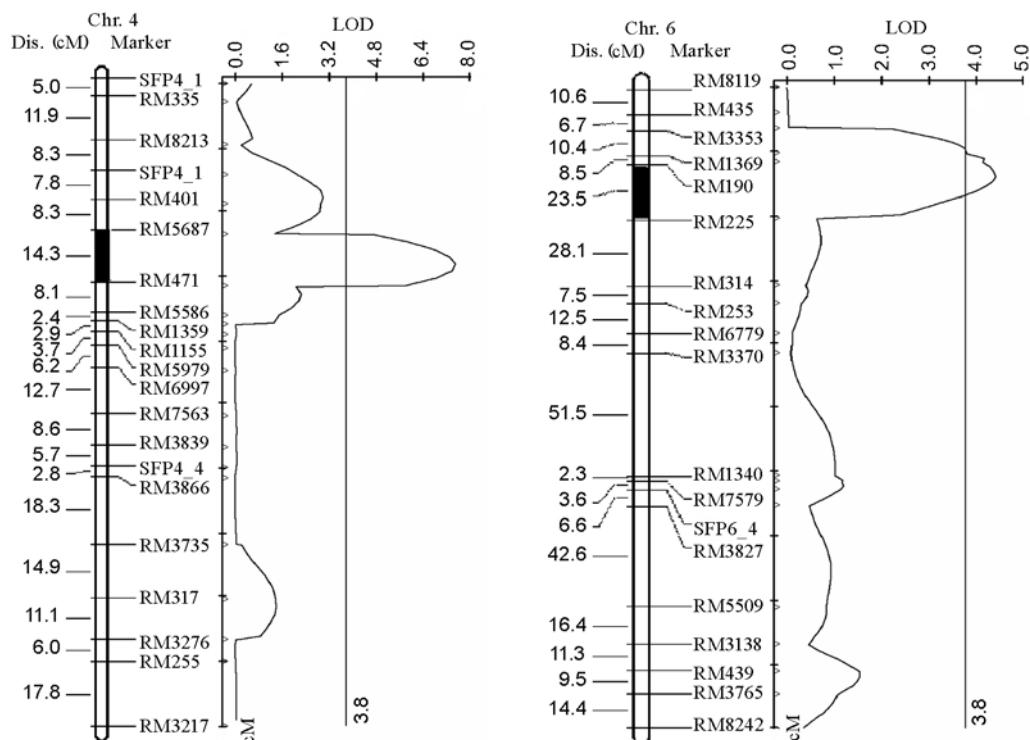


Fig. 2. Chromosomal locations of the QTLs associated with pollen fertility of rice grown under high temperature stress at the flowering stage identified in the 996/4628 recombinant inbred line population.

The solid curve denotes the LOD scores of the putative QTL detected with a step of 2 cM by using composite interval mapping method.

(Fig. 2, Table 1). *qPF4* on chromosome 4 is flanked by the markers RM5687 and RM471, with a LOD score of 7.54 and explained 15.1% of the total phenotypic variation in PF (Table 1). The QTL *qPF6* on chromosome 6, is flanked by the markers RM190 and RM225, with a LOD score of 4.43 and explained 9.31% of the total phenotypic variation in PF (Table 1). The alleles from cultivar 996 were associated with a 7.15% and 5.25% increases of pollen fertility percentage for plants subjected to HTS in the field (Table 1).

Each of the four molecular markers linked to PF heat tolerance was stable, with the clear different bands in the 8% PAGE gels of PCR products between the two parents. The band sizes of the markers RM5687, RM471, RM190 and RM225 were consistent with previous report, with each of approximate 245–260 bp, 190–200 bp, 140–150 bp and 190–200 bp, respectively in present study.

DISCUSSION

Previously, considerable studies on HTS focused on the analysis of agronomical and physiological characteristics of the trait, including the spikelet sterility, seed setting, and anther dehiscence (Satake and Yoshida 1978; Matsui et al, 1999, 2000, 2001, 2002, 2003; Prasad et al, 2006; Jagadish et al, 2007). In the recent years, about 12 QTLs have been identified using different mapping populations derived from indica/japonica crosses except for Chen's report (Cao et al, 2002, 2003; Zhu et al, 2005; Zhao et al, 2006; Chen et al, 2008; Zhang et al, 2008). Nevertheless, the accuracy of QTL mapping, in which the heat tolerance were evaluated by seed setting percentage (SSP) or 1000-grain weight, was influenced by the hybrid sterility in indica/japonica hybrids. In present study, the QTL associated the pollen fertility under high temperature stress, which detected in recombinant inbred population derived from indica/indica cross, could stand the exact tolerant ability to HTS in rice.

In the same field experiment, two QTL associated with seed setting percentage under HTS, named *SSP_{F4}* and *SSP_{F10}*, were detected on the chromosomes 4 and 10 (unpublished). *SSP_{F4}* which is

flanked by the markers RM5687 and RM471 on chromosome 4 explained 21.32% of the total phenotypic variation in SSP in the field. The allele from cultivar 996 was associated with a 9.11% increase of SSP for plants subjected to HTS in the field. It is inferred that *qPF4* and *SSP_{F4}* are the same QTL conferring high temperature stress tolerance in the rice cultivar 996. Previously, Zhu et al (2005) reported a heat tolerance QTL located between the two RFLP markers C1100 and R1783 on chromosome 4. This QTL was identified based on the grain weight heat sensitivity index [GWHSI=(grain weight under optimum temperature–grain weight under high temperature)/grain weight under optimum temperature×100]. GWHSI was measured for rice exposed to high temperatures from 5 days after initial heading to maturity. The other QTL located on chromosome 4 flanked by SSR markers RM241 and RM1018 was detected based on the SSP of plants treated with high temperature during the booting stage (Zhao et al, 2006). However, the location of the QTL mapped on chromosome 4 in the current study differs from that of the two QTLs described in the previous studies based on the marker information reported by McCouch et al (2002). It is indicated that *qPF4* harbored in the highly HTS tolerant cultivar 996 could be useful for developing rice cultivars with increased tolerance to HTS.

On chromosome 6, where locates the QTL of pollen fertility tolerant to HTS (*qPF6*) in this study, no QTL conferring seed set percentage were detected (unpublished). It is suggested that the genetic factors of seed set percentage (SSP) and pollen fertility (PF) tolerance to HTS were not entirely consistent. On one hand, SSP decrease may be caused by many factors besides pollen sterility, including disruption of anther dehiscence, poor pollen germinating and inhibited pollen tube growth under high temperature stress. On the other hand, for some lines with lower pollen fertility percentage under high temperature stress, however their stigma could be pollinated by normal germinated pollen from other florets, thus resulting in unobvious decrease in seed set.

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REFERENCES

- Cao L Y, Zhao J G, Zhan X D, Li D L, He L B, Cheng S H. 2003. Mapping QTLs for heat tolerance and correlation between heat tolerance and photosynthetic rate in rice. *Chinese J Rice Sci*, 17 (3): 223-227. (in Chinese with English abstract)
- Cao L Y, Zhu J, Zhao S T, He L B, Yan Q C. 2002. Mapping QTLs for heat tolerance in a DH population from indica-japonica cross of rice (*Oryza sativa*). *J Agric Biotech*, 10(3): 210-214. (in Chinese with English abstract)
- Chen L Y, Tang W B, Liu G H, Deng H B, Chen S, Lei D Y, Zhang G L. 2006. Breeding of new high-yielding early hybrid rice combination Luliangyou 996. *Hybrid Rice*, 21(2): 24-26. (in Chinese with English abstract)
- Chen Q Q, Yu S B, Li C H, Mou T M. 2008. Identification of QTLs for heat tolerance at flowering stage in rice. *Sci Agric Sin*, 41(2): 315-321. (in Chinese with English abstract)
- Chen X, Temnykh S, Xu Y, Cho Y G, McCouch S R. 1997. Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). *Theor Appl Genet*, 95: 553-567.
- Churchill G A, Doerge R W. 1994. Empirical threshold values for quantitative trait mapping. *Genetics*, 138: 963-971.
- International Rice Genome Sequencing Project. 2005. The map-based of the rice genome. *Nature*, 436: 793-800.
- International Rice Research Institute. 2007. Coping with climate change. Available <http://www.irri.org/publications/today/pdfs/6-3/10-15.pdf>. *Rice Today*, 6(3): 10-15.
- Jagadish S V K, Craufurd P Q, Wheeler T R. 2007. High temperature stress and spikelet fertility in rice. *J Exp Bot*, 58: 1627-1635.
- Jeremy D E, Jaroslav J, Megan T S, Ambika B G, Liu B, Hei L, David W G. 2008. Development and evaluation of a high-throughput, low-cost genotyping platform based on oligonucleotide microarrays in rice. *Plant Meth*, 4: 13.
- Lincoln S, Daley M, Lander E. 1992. Constructing genetic maps with MAPMAKER/EXP 3.0, 3rd edn. Whitehead Institute Technical Report, Cambridge.
- Luo L H, Liu G H, Xiao Y H, Tang W B, Chen L Y. 2005. Influences of high-temperature stress on the fertility of pollen, spikelet and grain-weight in rice. *J Hunan Agric Univ (Nat Sci)*, 31(6): 593-596. (in Chinese with English abstract)
- Matsui T, Kagata H. 2003. Characteristics of floral organs related to reliable self pollination in rice (*Oryza sativa* L.). *Ann Bot*, 91: 473-477.
- Matsui T, Omasa K, Horie T. 1999. Mechanism of anther dehiscence in rice (*Oryza sativa* L.). *Ann Bot*, 84: 501-506.
- Matsui T, Omasa K, Horie T. 2000. High temperature at flowering inhibit swelling of pollen grains, a driving force for thecae dehiscence in rice (*Oryza sativa* L.). *Plant Prod Sci*, 3: 430-434.
- Matsui T, Omasa K, Horie T. 2001. The difference in sterility due to high temperatures during the flowering period among japonica rice varieties. *Plant Prod Sci*, 4: 90-93.
- Matsui T, Omasa K. 2002. Rice (*Oryza sativa* L.) cultivars tolerant to high temperature at flowering: anther characteristics. *Ann Bot*, 89: 683-687.
- Matsushima S, Ikewada H, Maeda A, Honda S, Niki H. 1982. Studies on rice cultivation in the tropics. 1. Yielding and ripening responses of the rice plant to the extremely hot and dry climate in Sudan. *Jpn J of Trop Agric*, 26: 19-25.
- McCouch S R, Teytelman L, Xu Y, Lobos K B, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L. 2002. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res*, 9: 199-207.
- Prasad P V V, Boote K J, Allen L H Jr, Sheehy J E, Thomas J M G. 2006. Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high temperature stress. *Field Crops Res*, 95: 398-411.
- Saghai Maroof M A, Biyashev R M, Yang G P, Zhang Q, Allard R W. 1994. Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. *Proc Nat Acad Sci USA*, 91(12): 5466-5470.
- Satake T, Yoshida S. 1978. High temperature induced sterility in indica rices at flowering. *Jpn J Crop Sci*, 47: 6-17.
- Temnykh S, Park W D, Ayres N, Cartinhour S, Hauck N, Lipovich L, Cho Y G, Ishii T, McCouch S R. 2000. Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theor Appl Genet*, 100: 697-712.
- Wang S C, Basten C J, Gaffney P, Zeng Z B. 2007. Windows QTL Cartographer 2.0 User Manual. Bioinformatics Research Center, North Carolina State University, Raleigh, North Carolina.
- Wu K S, Tanksley S D. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. *Mol Gen Genet*, 241: 225-235.
- Xia M Y, Qi H X. 2004. Effects of high temperature on the seed setting percent of hybrid rice breed with four male sterile lines. *Hubei Agric Sci*, (2): 21-22. (in Chinese)
- Xu Y B, Shi C H, Sheng Z D. 1989. Effects of high temperature on seed set percentage in early-season rice. *J Zhejiang Agril Univ*, (2): 51-54. (in Chinese)
- Yang H C, Huang Z Q, Jiang Z Y, Wang X W. 2004. High temperature damage and its protective technologies of early and middle season rice in Anhui province. *J Anhui Agric Sci*, 32(1):

3-4. (in Chinese)

Zeng Z B. 1994. Precision mapping of quantitative trait loci. *Genetics*, 136: 1457-1468.

Zhang T, Yang L, Jang K F, Huang M, Sun Q, Chen W F, Zheng J K. 2008. QTL mapping for teat tolerance of the tassel period of rice. *Mol Plant Breed*, 6(5): 867-873. (in Chinese with English abstract)

Zhao Z G, Jiang L, Xiao Y H, Zhang W W, Zhai H Q and Wan J M. 2006. Identification of QTLs for heat tolerance at the booting stage in rice(*Oryza sativa* L.). *Acta Agron Sin*, 32(5): 540-644. (in Chinese with English abstract)

Zhu C L, Xiao Y H, Wang C M, Jiang L, Zhai H Q, Wan J M. 2005. Mapping QTL for heat-tolerance at grain filling stage in rice. *Rice Sci*, 12(1): 33-38.

Zou J, Liu A L, Chen X B, Zhou X Y, Gao G F, Wang W F, Zhang XW. 2009. Expression analysis of nine rice heat shock protein genes under abiotic stresses and ABA treatment. *J Plant Physiol*, 166(8): 851-861.