A Dominant Major Locus in Chromosome 9 of Rice (Oryza sativa L.) Confers Tolerance to 48 °C High Temperature at Seedling Stage

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Abstract

In an earlier greenhouse screening, we identified a local indica cultivar HT54 tolerant to high temperature at both seedling and grain-filling stages. In this study, we develop an optimized procedure for fine assessment of this heat tolerance. The results indicated that HT54 seedlings could tolerate high temperature up to 48 °C for 79 h. The genetic analysis of F1 and F2 offspring derived from the cross between HT54 and HT13, a heat-sensitive breeding line, reveals that the heat tolerance of HT54 was controlled by a dominant major locus, which has been designated as OsHTAS (Oryza sativa heat tolerance at seedling stage). This locus was mapped on rice chromosome 9 within an interval of 420 kb between markers of InDel5 and RM7364. The determined candidate ZFP gene has been confirmed to be cosegregated with a single nucleotide polymorphism (SNP) developed PCR-restriction fragment length polymorphism (RFLP) marker RBsp1407 in its promoter region. Another heat tolerance-associated SNP was identified in the first intron of its 5′-untranslated region. The existence of these SNPs thereby indicated that the OsHTAS locus contains at least two alleles. We named the one from HT54 as OsHTASa and the one from HT13 as OsHTASb. Further dynamic expression analysis demonstrated that OsHTASa was actively responsive to 45 °C high temperature stress compared with the OsHTASb allele.

Key words: cosegregation, gene mapping, OsHTAS, PCR-RFLP, RBsp1407

Due to accelerated global warming, high temperature damages global rice production more frequently (Hall 2001) than in past decades (Easterling et al. 1997; Jones et al. 1999). In rice, a high temperature, 5 °C beyond threshold level, at flowering can induce floret sterility and consequently high yield losses (Osada et al. 1973; Stake and Yoshida 1978; Matsushima et al. 1982), with occasionally up to 80% loss (Li 2003). Much effort has been made to carry out molecular mapping of heat-tolerance quantitative trait loci (QTLs) at booting, flowering, and grain filling to ripening stages in rice. A total of 52 main effect QTLs and 25 epistatic ones capable of explaining 2.27–50.11% of phenotypic variance have been identified on loci covering all 12 chromosomes (Cao et al. 2002; Cao et al. 2003; Zhu et al. 2005; Zhu et al. 2006; Zhao et al. 2006; Chen et al. 2008; Kui et al. 2008; Zhang et al. 2008; Jagadish et al. 2010; Pan et al. 2011; Xiao et al. 2011; Ye et al. 2012). The most significant heat-tolerance QTL that can explain up to 50% of phenotype variance is from Kasalath, an indica landrace cultivar from India (Zhu et al. 2006). Similarly, a QTL that can explain the largest phenotype variance for spikelet fertility/seed setting is contributed by IR64, an elite indica cultivar from International Rice Research Institute (IRRI, Manila, Philippines) (Cao et al. 2002). However, genes that govern the heat tolerance with qualitative effects on both plant growth and development have not yet been identified.

The early stage of seedling growth is of the utmost importance in stand establishment. Very hot weather at this critical stage is undoubtedly injurious to rice plants. To cope with heat...
stress, plants have adopted various physiological mechanisms, including maintenance of membrane stability, scavenging of reactive oxygen species, production of antioxidants, accumulation and adjustment of compatible solutes, induction of mitogen-activated protein kinase and calcium-dependent protein kinase cascades, and most importantly, chaperone signaling and transcriptional activation (Bohnert et al. 2006; Wahid et al. 2007; Barnabás et al. 2008). Many of these mechanisms take place at vegetative phases, including the seedling stage (Stone 2001) and enable plants to thrive under heat stress (Wahid et al. 2007). Although great progress has been made in understanding these physiological mechanisms, further studies focused on phenotyping, mapping, and cloning of novel loci modulating seedling heat tolerance are imperative.

In a greenhouse screening conducted at the Rice Research Institute of Guangdong Academy of Agricultural Sciences, a local indica cultivar HT54 was identified to tolerate high temperature of 45 °C for 5–7 days at both seedling and grain-filling stages. In this study, we aimed to develop a standard procedure for the evaluation of heat tolerances and then map this novel heat-tolerant locus using simple sequence-repeat length polymorphism (SSLP, abbreviated as SSR, thereafter) markers. Gene mapping and associated genetic analysis will provide a base for further molecular cloning and functional dissection of the candidate gene.

Materials and Methods

Plant Materials

The plant materials used in this study were HT54, an indica breeding line with heat tolerance possibly inherited from its parental variety Guang-Lu-Ai No 4, and HT13, a heat-sensitive breeding line having a phylogenetic relationship to an indica cultivar of 05-Zhan. Both lines were collected from the rice production area of southern China and their main agronomic traits are listed in Table 1.

High Temperature Treatment

In order to precisely conduct gene mapping and associated genetic analysis, an optimized procedure for assessment of heat tolerance was developed using HT54 and HT13 seedlings as samples. The seedlings were transferred in soil and grown up to the 2.5- to 3.5-leaf stage. After collection of leaf samples (for DNA extraction and mapping population construction afterwards) and recovery for 2 days, these plants were transferred into a growth cabinet. The temperature was gradually increased from 33 to 48 °C over 5 h and maintained at 48 °C for 79 h with relative humidity (RH) of 75%. For the seedlings that were cultured in Yoshida culture solution, the treatment time was increased to 84 h. Light and dark were rotated every 12 h throughout the experiment. Planting density, tray soil weight, nutrition solution amount, fertilizing level, and watering amount were set at fixed levels. The size of the tray used for seedling cultivation was 43 × 33 × 10 cm with 11 rows and 18 plants per row. Two rows of heat-tolerance plants grown in the tray periphery were used to protect the experimental samples from marginal overheating. All high temperature treatments applied in this study were carried out in PRX-1000B growth cabinet purchased from Ningbo China. Considering the limited space inside cabinet and the existence of a marginal effect, the high temperature treatment was done one tray at a time.

Genetic Analysis and Mapping Population Construction

The heat-sensitive HT13 was used as female parent and crossed with the heat-tolerant HT54 in the experimental farm of Rice Research Institute of Guangdong Academy of Agricultural Sciences in autumn of 2008. The resultant F1 plants were screened at seedling stage using SSR markers. Each panicle on the true F1 plants was bagged to prevent outcrossing, and their selfing seeds were then collected and used to produce the F2 population.

The same batches of high temperature-treated F2 plants were used for both genetic analysis and mapping population construction. The parental plants of HT54 and HT13 served as controls. The checkpoint for termination of treatment was 79 h, at which time HT54 parent plants remained normal, but HT13 plants had completely withered and died. The treated plants were then transferred from the cabinet to the normal growth condition and allowed to recover for 5 days. Treated plants were scored as tolerant or sensitive based on whether their reaction was similar to HT54 or to HT13. The inheritance behavior of heat tolerance in HT54 was determined. Chi-square test was applied to check the goodness-of-fit of F2 segregation ratio. The F1 plants plus control plants from both parents were treated with high temperature separately for judging whether heat tolerance in HT54 is inherited as dominant or recessive.

The mapping population was constructed using the extreme recessive segregants. The DNA samples were prepared using the leaf tissue of these segregants collected 2 days before the high temperature treatments. The DNA samples from both parental lines were used as controls.

Molecular Mapping of High Temperature Tolerance in HT54

The molecular mapping of the heat tolerance in HT54 was performed via SSR tagging (Zhang et al. 1994; Chen et al.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Plant height (cm)</th>
<th>Growth duration (days)</th>
<th>Panicle number/plant</th>
<th>Grain number/plant</th>
<th>Seed setting rate (%)</th>
<th>Thousand grain weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT54</td>
<td>85.38 ± 2.21</td>
<td>101.0 ± 1.20</td>
<td>6.47 ± 1.06</td>
<td>132.98 ± 7.62</td>
<td>85.26 ± 5.76</td>
<td>30.83 ± 0.45</td>
</tr>
<tr>
<td>HT13</td>
<td>89.83 ± 4.15</td>
<td>125.0 ± 1.30</td>
<td>5.87 ± 1.60</td>
<td>111.73 ± 6.79</td>
<td>85.63 ± 9.00</td>
<td>22.15 ± 0.09</td>
</tr>
</tbody>
</table>

Table 1 Agronomic traits of HT54 and HT13
1997; Temnykh et al. 2000). A total of 2304 SSR markers available in the genomic database (http://www.gramene.org) and covering the whole rice genome were selected for the parental survey. Of these, 322 SSR markers polymorphic between the parents were used to detect differences between two DNA pools, each of which contained either 10 sensitive or 10 tolerant samples randomly selected from the first batch of temperature-treated F_2 offspring. The SSR markers polymorphic between the two pools were confirmed to be associated with recessive gene using all the F_2 plants.

The further density increased marker analysis was carried out using SSR markers available within a linkage group. Two self-designated insertion–deletion markers, InDel3 and InDel5, were added to this analysis. The sequences of primers used for amplifying these InDel markers were InDel3F: 5′-GTTTGCGACA TTGGAGGGCTTC-3′, InDel3R: 5′-AATGCTTGAGTATGCTAGGTA-3′; and InDel5F: 5′-TCTGGAGAGTTTCTGATGTG-3′, InDel5R: 5′-CAGAAGG TGTAAGCAACTCTTGT-3′. DNA extraction and PCR amplification were performed as described by Chen et al. (1997). The amplified products were separated by electrophoresis on 3.5% agarose gel or 4.0% polyacrylamide gels, and the band patterns were visualized under ultraviolet (UV) light after staining with ethidium bromide or detected by silver staining (Panaud et al. 1996). Segregating data were analyzed using software MapMaker 3.0 (Lander et al. 1987). Linkage maps were obtained using software MapDraw 2.1 (Liu and Meng 2003).

**PCR-RFLP Analysis**

PCR-RFLP analysis was performed on the basis of the procedure described by Toda et al. (2002). DNA was extracted from fresh leaves of recombinant plants existing in the F_2 mapping population after density increased marker analysis. DNA samples from parental plants were used as controls. The target sequence of the mutant site detected in the candidate gene was amplified from the above DNA samples and digested with the mutant site-corrresponding restriction enzyme. Digestions were fractionated on 3% agarose gel containing Gelred and photographed under UV light.

**RNA Isolation and RT-PCR Analysis**

Total RNA was isolated from fresh leaves of both parental plants at seedling stage using Trizol reagent (Invitrogen), according to manufacturer’s instructions. Prior to leaf collection, the seedlings cultured in Yoshida solution were challenged with high temperature of 45 °C in the growth room under light and 75% RH. An equal amount of leaf tissue from each batch of plants per parent was sampled at 0, 1, 2, 4, 6, 8, 10, and 12 h. First-strand complementary DNA (cDNA) synthesis was carried out using the M-MLV first-strand synthesis system (Promega). The fragments of coding sequence were amplified from the cDNAs using the candidate gene-specific primers. The semi-quantitative RT-PCR was performed under the following conditions: pre-denaturation at 94 °C for 4 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C in 25 µL of reaction mixture containing 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, and 1.5 mM MgCl_2), 0.2 mM dNTPs, 0.2 µM of each primer, 1 unit of Taq polymerase. Actin I genes amplified from each parent under the same running conditions with 25 cycles were used as references. All PCR reactions were repeated using at least three independent samples. PCR products were fractionated on 1% agarose gel containing Gelred and photographed under UV light.

**Results**

**Optimization of Heat-Tolerance Assessment Procedure**

HT54 is a semi-dwarf cultivar with short growth duration, broad flag leaf, and acceptable yield traits compared with HT13. In order to optimize the procedure for heat stress assessment, several key factors that may influence the assessment results were calibrated in advance using parental seedlings as samples. Optimized factors included treatment temperature, treatment time, seedling stage, and culturing method. The data show that the proper temperature for clearly distinguishing tolerant and sensitive phenotypes between HT54 and HT13 was 48 °C (Figure 1, row III). The best treatment stage was when seedlings grown up to 2.5 to 3.5 leaves. Both Yoshida solution and soil culturing methods were practicable for the assessment, but different lasting times for the temperature treatment were required to enable them to achieve the best effect. The lasting time for the Yoshida solution culturing method was 84 h (Figure 1A, column I), whereas for the soil culturing method, it was 79 h (Figure 1A, column II).

As expected, the HT54 seedlings treated with temperatures of 42, 45, and 48 °C according to the optimized heat stress procedure all survived. In contrast, the same procedure-treated HT13 seedlings completely withered or died under temperatures of 45 and 48 °C (Figure 1A, row C). The maximum temperature that HT54 seedlings could tolerate under the present experimental condition was 48 °C, which was at least 3 °C higher than that for HT13 seedlings.

**Inheritance of Heat Tolerance in HT54**

The dominant–recessive analysis of heat tolerance was conducted in the F_1 generation. Seedlings were cultured in Yoshida solution, and two parental cultivars were used as tolerant and sensitive controls. After challenging with 48 °C for 84 h, all F_1 seedlings survived (Figure 1B), indicating an identical phenotype to the heat-tolerant parent HT54 but different phenotype from the heat-sensitive parent HT13. These results confirm that heat tolerance in HT54 was inherited as completely dominant.

The high temperature treatment for analyses of heat tolerant to sensitive segregation ratio was performed using five batches of F_2 plants. Soil-cultured seedlings were treated at 2.5-leaf stage, and two parental cultivars were used as tolerant and sensitive controls. Of 744 F_2 plants, 548 were as heat tolerant as HT54 and 196 were as heat sensitive as
Figure 1. High temperature stress test of HT54 and HT13 and their F₁ seedlings at 2.5- to 3.5-leaf stage. (A) High temperature stress test of HT54 and HT13 seedlings at 42 °C (top row), 45 °C (middle row), and 48 °C (bottom row). Left column: seedlings that were cultured in Yoshida solution and recovered for 1 day after 84 h treatment; right column: seedlings that were cultured in soil and recovered for 5 days after 79 h treatment. The light and dark were rotated once per 12 h, and the RH was set at 70%. Other factors including planting density, fertilizing level, watering amount, tray soil weight, and nutrition solution volume for each batch of materials in a seedling-culturing method were set at fixed levels throughout the experiment. (B) High temperature stress test of F₁ seedlings at 48 °C for 1 day. Temperature treatment procedures and seedling-culturing method were the same as in (A).
HT13 (Table 2). The segregation of heat tolerant to sensitive fits the 3:1 ratio of Mendel genetic law with probability 0.30–0.20 after chi-square test (Table 2). These results thereby demonstrated that heat tolerance in HT54 was controlled by a major locus. We named this locus OsHTAS (Oryza sativa heat tolerance at seedling stage).

Molecular Mapping of High Tolerance in HT54

The primary linkage group analysis of OsHTAS was performed using two DNA pools, composed of 10 resistant and 10 susceptible F2 plants, as samples. Among 322 SSR polymorphic markers detected, only RM444 showed band-pattern difference between the two DNA pools. Data deposited in GeneBank (http://www.ncbi.nlm.nih.gov) indicated that RM444 is located in the long arm of chromosome 9.

Further, density increased marker analysis was carried out using the mapping population developed from 130 extreme recessive F2 segregants, which were sensitive to high temperature. A total of 12 polymorphic SSR markers (out of 250) plus two self-designated InDel markers (InDel3 and InDel5) spanning a 18.45-Mb genomic region from RM367 to RM242 were used for this purpose. The OsHTAS locus was located between markers InDel5 and RM7364 (Figure 2A). The map distance between OsHTAS and two closely linked markers was 2.5 and 1.7 cM, respectively (Figure 2A). The physical distance between the markers InDel5 and RM7364 was 420.0 kb (Figure 2A).

Determination of Candidate Gene within the Mapped Genomic Region

Further analysis revealed that this 420-kb sequence contains 23 cDNA-producing genes, including ubiquitin-conjugating enzyme (LOC_Os09g15170) and zinc finger family protein (ZFP) (LOC_Os09g15430) (Figure 2A). Because these two genes have been associated with heat stress response (Huang et al. 2008; Nigam et al. 2008; Yang et al. 2008), we considered them as primary candidates for the dominant OsHTAS locus.

To address which of the two genes corresponds to the dominant OsHTAS locus present in HT54, we compared sequencing data amplified from HT54 and HT13 (Supplementary Table S1). The results indicated that of the two genes, the dominant allele of ZFP from HT54 had an extra G at the −1395th nucleotide in its first intron of 5′-untranslated region and a single base substitution of T to G at the −3060th nucleotide in its promoter region (Figure 2A). The detection of these 2 single nucleotide polymorphisms (SNPs) thereby suggested that the detected ZFP might be the OsHTAS locus.

To further confirm the above results, a PCR-RFLP analysis was established. The sequence corresponding to the SNP site in the promoter region of the ZFP from HT13 is the recognition site (TGT−3060ACA) of Bsp1407 restriction enzyme. The single base substitution of T−3060 to G−3060 occurring in HT54 eliminated this restriction recognition site. The polymorphism of this restriction recognition site of Bsp1407 between HT54 and HT13 was then designated as a PCR-RFLP marker, called RBsp1407. The PCR-RFLP analysis was performed on the three recombinant plants of the F2 recessive mapping population after screening with the two closely linked markers of InDel5 and RM7364. The two parental cultivars were used as control. The primers used were BspF: 5′-CCATCCAAACACGCCCTAA-3′ and BspR: 5′-ATTGCCCTTGTGCTATGGT-3′. When subjected to Bsp1407 digest, all PCR products amplified from these three recombinant plants were cut into two fragments of 422 and 158 bp, which was identical to the digest of PCR product amplified from the heat-sensitive parent HT13 (Figure 2B). These results confirmed that this PCR-RFLP marker cosegregated with the heat-sensitive phenotype. Thus, the candidate ZFP gene based on DNA sequencing indeed corresponds to the OsHTAS locus. We tentatively name the allele of ZFP from HT54 as OsHTASa and that from HT13 as OsHTASb.

Dynamic Expression of OsHTASa and OsHTASb under Heat Stress Conditions

The dynamic expressions of OsHTASa and OsHTASb were monitored under heat stress conditions via semi-quantitative RT-PCR. The primers used for this analysis were sqZfpF: 5′-TGCACACAT GACGAAGGGAATA-3′ and sqZfpR: 5′-CAATCATCAGAAGGACGCAATC-3′. The level of rice actin1 transcripts was used as internal reference. The temperature for heat stress was set at 45 °C. Fresh leaf messenger RNAs (mRNAs) were isolated at 0, 1, 2, 4, 6, 8, and 12 h after heating. The levels of OsHTASa mRNA transcripts experienced a decline after the first 2 h, climbed to a peak value at 6 h, declined again from 8 to 10 h, and finally rose slightly at 12 h. However, the dynamic expression of OsHTASb under the same heat stressing conditions appeared as a steady decline across the time interval. The different expression of the OsHTAS locus between HT54

| Table 2 Segregation ratio of tolerance to sensitive detected in F2 seedlings after challenging with high temperature |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Experiments    | Total | Survival | Dead | χ² (3:1) | P (α = 0.05) |
| I              | 150   | 106     | 44   | 1.509    | 0.30–0.20     |
| II             | 152   | 112     | 40   | 0.135    | 0.80–0.70     |
| III            | 144   | 109     | 35   | 0.037    | 0.90–0.80     |
| IV             | 136   | 104     | 32   | 0.163    | 0.70–0.50     |
| V              | 162   | 117     | 45   | 0.623    | 0.50–0.30     |
| Total          | 744   | 548     | 196  | 0.708    | 0.30–0.20     |
and HT13 thereby indicated that the dominant allele of OsHTAS<sup>a</sup> from HT54 was actively responsive to the heat stress.

**Discussion**

Precise and accurate phenotyping is the key step to ensuring success in gene/locus mapping. In this study, we developed an optimized procedure for heat-tolerance assessment at the rice seedling stage. The optimum temperature and treatment time were 48 °C and 79 h (for soil culturing method) or 84 h (for Yoshida solution culturing method). The suitable growth stage for heat stress was when seedlings had grown up to the 2.5- to 3.5-leaf stage. Other relevant indices were set at fixed levels throughout the experiment. Tolerant seedlings from HT54 parents or F<sub>1</sub> and F<sub>2</sub> hybrids all survived, whereas sensitive seedlings from HT13 parents and F<sub>2</sub> hybrids withered and died.

Because of the optimization procedure, we can clarify the inheritance behavior of the OsHTAS locus that confers heat tolerance at seedling stage for HT54 and consequently map it onto chromosome 9 (Figure 2A). The candidate gene thus determined may be a ZFP-encoding gene with allele OsHTAS<sup>a</sup> from HT54 and allele OsHTAS<sup>b</sup> from HT13. Interestingly, Zhu et al. (2006) also mapped a heat-tolerance QTL for accumulation of amylose content onto chromosome 9 at a locus upstream of OsHTAS. However, the physical positions of amplification fragments on chromosome 9 did not overlap, but were 1.1 Mb apart from each other according to the TIGR database (http://rice.plantbiology.msu.edu/).

Additionally, there is accumulated evidence that heat shock proteins (HSPs) are involved in the acquired thermo tolerance of higher plants (Liu and Shono 1999; Burke et al. 2000; Hong and Vierling 2000; Queitsch et al. 2000; Iba 2002; Moriarty et al. 2002; Sanmiya et al. 2004; Chang et al. 2007; Barnabás et al. 2008; Sarkar et al. 2009). In rice, a collection of
321 HSP-coding sequences annotated from the Nipponbare genome are currently available in the GeneBank, EMBL, and DDBJ databases (http://www.ncbi.nlm.nih.gov). However, none of these are located within the interval between InDel5 and RM7364. Huang et al. (2008) cloned four cold-induced rice A20/AN1-ZFP genes and found that overexpression of gene ZFP177 in tobacco confers tolerance on transgenic plants to both low and high temperature stress. Although this gene is also located in the rice chromosome 9, its locus falls 9 Mb downstream of RM7364. Therefore, to the best of our knowledge, the mapped and cloned OsHTASv allele from HT54 in this study is a novel allele controlling heat tolerance at least at the rice seedling stage. Further functional analysis and elucidation of the heat-tolerance mechanism is in progress.

A recent study has shown that annual mean day and night temperature has increased globally by 0.35 and 1.13 °C, respectively, for the period 1979 and 2003, and rice grain yields have declined by 10% for each 1 °C increase in night temperature (Peng et al. 2004). The trend of this global climate warming will continue (Salinger 2005; IPCC 2007) and constitute a more and more serious menace for rice production (Battisti and Naylor 2009). In 2003, a heat wave with temperatures above 38 °C for 20 days occurred in rice production regions of southern China. This resulted in failed crops and created disaster areas across 530 000 and 500 000 ha in two provinces Anhui and Hubei, respectively (Yang et al. 2004; Xia and Qi 2004). In addition, it is estimated that there are approximately 4 000 000 ha of rice under potential threat of high temperature worldwide, and the area covers 14 main rice production areas in Asia, Africa, and United States (Mutsuo 1990). Therefore, new varieties with improved heat tolerance are a desperate need in rice production. The identification of the OsHTASv gene and its cosegregating marker RBsp1407 in this study will provide a basis for effective strategies in improving heat tolerance in rice plants.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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Oryza sativa

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