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ORIGINAL ARTICLE

Identification and Fine Mapping of a Major QTL, *TT1-2*, That Plays Significant Roles in Regulating Heat Tolerance in Rice

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Abstract

Global warming threatens many aspects of human life, including a reduction in crop yields, and breeding heat-tolerant crops is a fundamental way to help address this challenge. As food for more than half of the global population, rice (*Oryza sativa*) has always been a popular research material in plant science. Breeding heat-tolerant rice using genes affording thermotolerance is a fundamental way to address this challenge. In this study, a major QTL, *TT1-2*, was found to regulate heat tolerance in rice; this QTL was controlled by a single dominant gene. Using $F_{2:3}$ populations, we narrowed *TT1-2* to a 26.0-kb region containing three putative genes, one of which encodes an α2 subunit of the 26S proteasome. This gene was considered the *TT1-2* candidate, and the *TT1* gene involved in rice heat tolerance was present at this locus. Further analysis showed that the amino acid sequence of the *TT1-2* gene had one amino acid difference: arginine (R)-99 was changed to histidine (H), which leads to the formation of a normal alpha-helix. Moreover, through marker-assisted selection and conserved breeding selection, we developed a new male-sterile line, Zhehang 10A, which had a high outcrossing rate, good quality, and strong heat tolerance. We then used Zhehang 10A as the female parent and the restorer line Fuhui 1586 as the male parent and bred a new hybrid, Zhehangyou 1586, which showed high yield, good quality, and strong heat tolerance.

Keywords Rice (*Oryza sativa* L. subsp. *indica*) · Mapping · Cloning · Heat tolerant · Breeding application

Introduction

Changes in air temperature and precipitation have affected the global climate scenario, wherein the global surface temperature has increased by an average of 0.85 °C during the past century, while changes in precipitation have varied geographically (Lawas et al. 2019). Heat stress leads to severe crop yield losses and reduced milling quality (Mba et al. 2012) and is predicted to cause food crises in the future (Trnka et al. 2014).

Rice (*Oryza sativa* L.) is one of the most important food crop species worldwide and requires large quantities of water during its growth cycle (Wu et al. 2009). Heat stress negatively influences crop growth and yield (Kilasi et al. 2018). Several studies have reported the use of a potential transgenic approach in enhancing heat stress tolerance in rice (Wu et al. 2009; Kilasi et al. 2018; El-Esawi and Alayafi 2019). Moreover, heat tolerance genes such as *DPB3-1*, which in *Arabidopsis* encodes a positive transcriptional regulator, have been reported in rice, and enhanced heat stress tolerance without growth retardation has also been reported in rice (Sato et al. 2016). *OsHTAS* plays a positive role in heat tolerance at the seedling stage and encodes a ubiquitin ligase localized to the nucleus and cytoplasm (Liu et al. 2016). *OsHTAS* is located at a major quantitative trait locus for thermotolerance. *OsTT1* encodes an α2 subunit of the 26S proteasome, which is involved in the degradation of ubiquitinated proteins, and ubiquitylome analysis has indicated that *OsTT1* protects cells from heat stress through more efficient elimination of

Key Message A major QTL, *TT1-2*, was found to regulate heat tolerance in rice, and a single amino acid difference in the *TT1-2* gene could affect the formation of a normal alpha-helix. A new hybrid combination, Zhehangyou 1586, was bred using a developed molecular marker linked to *TT1-2*.

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cytotoxic denatured proteins and more effective maintenance of heat-response processes compared with those of other genes (Li et al. 2015). However, developing more stress-tolerant rice varieties is currently essential for use in breeding strategies to overcome the adverse effects caused by heat stress and to meet the increasing global population demands.

In this study, using a bin map converted from the ultrahigh-quality physical map associated with heat tolerance, we identified a heat tolerance gene, *TT1-2*, by map-based cloning. Molecular cloning of *TT1-2* and phenotypic analysis of the *TT1-2* gene were also performed. Moreover, through marker-assisted selection, we bred a new hybrid, Zhehangyou 1586, which showed high yield, good quality, and strong heat tolerance.

Materials and Methods

Plant Materials and Construction of the Mapping Population

The *indica* rice line Y1502 and the *japonica* rice lines Nipponbare, Xiushui 03, Zhehang 10A, and Fuhui 1586 were maintained at the Institute of Crop and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences.

In the summer of 2014, the rice cultivar Nipponbare was used as a pollen donor in crosses with Y1502. Twenty true F_1 hybrid seeds were sown at the Sanya Experimental Station in Hainan Province in the spring, and the resulting F_2 seeds were harvested. A total of 3464 individual plants of the F_2 population were planted at the Hangzhou Experimental Station in Zhejiang Province in the summer of 2015. A total of 3438 $F_{2:3}$ seeds were ultimately harvested, and a total of 804 non-heat-tolerant plants in the $F_{2:3}$ population were selected for fine mapping. The field experiment was established in accordance with a randomized plot design, with three plots per genotype. Zhehangyou 1586 and Tianxie 1 were planted in Fexi county of Anhui province in eight rows, and three plots were selected for investigation of agronomic traits.

All plants were planted in accordance with standard commercial practices, with spacing of 26.4 cm between rows and 13.3 cm within rows. The field management generally followed normal agricultural practices.

Plant Cultivation and Thermotolerance Identification

The rice seedlings used in this study were cultivated by hydroponic culture in Yoshida solution (pH 5.8). To break any possible dormancy, seeds were incubated at 42 °C for approximately 14 days. The seeds that had broken dormancy were soaked and pregerminated, and uniform germinated seeds were planted in 96-well plates whose bottoms were removed. The plates were placed on scaffolds in a container of water such that the seeds were partly immersed in the water, after which they were incubated for 1 day at 35 °C in the dark to encourage root growth. The rooted seeds were transferred to Yoshida solution and cultured at 28 °C and 50% relative humidity under a 13-h light/11-h dark photoperiod. The Yoshida solution was changed every 2 days. Heat treatment was used to minimize the effects of high-light stress and hydrophobic stress; a high relative humidity (> 80%) and low light intensity were used (50–80 μmol m^{-2} s^{-1}) (Hasanuzzaman et al. 2013). The specific method of heat treatment involved incubating 12-day-old (two-leaf stage) seedlings grown in hydroponic culture solution at 45 °C for 52 h, after which they were returned to normal conditions (28 °C) for 1 week for recovery. For genetic analysis and gene mapping, the specific heat treatment method was used.

The survival state of the seedlings was evaluated to determine their thermotolerance. For the survival rate analysis of Y1502, Nipponbare, Zhehangyou 1586, and Tianxie 1, 12-day-old seedlings were treated with 45 °C for 45 h followed by recovery at 28 °C for 2 weeks, and the number of plants in each line and their corresponding survival were recorded and used to calculate survival rates.

Polymerase Chain Reaction-Based Amplification and Marker Detection

Plant DNA was extracted from the frozen leaves of rice plants using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980), with minor modifications. For polymerase chain reaction (PCR)-based amplification of markers, each 20 μl reaction mixture involved 50 ng DNA, 5 μmol of each primer, 10× PCR buffer (100 mM Tris (pH 8·3), 500 mM potassium chloride (KCl), 15 mM magnesium chloride ($MgCl_2$), 2 μg of gelatin, each deoxynucleotide triphosphate at 250 μM, and 0.5 dynamic units (U) of Taq polymerase. Amplification was performed using the following program: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 60 °C (for Indel) or 55 °C (for SSR), and 2 min at 72 °C; and a final extension of 5 min at 72 °C. The amplified PCR products were resolved by electrophoresis on 8% polyacrylamide denaturing gels with silver staining for marker analysis (Panaud et al. 1996).

Bulked Segregant Analysis

Bulked segregant analysis was used to identify the markers linked to the target gene. A DNA pool was constructed from the DNA extracted from leaves of 15 non-heat-tolerant strains and 15 heat-tolerant strains selected randomly from the $F_{2:3}$ population. The SSR markers distributed in the rice genome were used to detect linkage, with DNA extracted from Y1502 and Nipponbare used as a control. The band type of the

markers linked with the no-heat-tolerance gene was the same as that in Nipponbare.

Molecular Mapping of the *TT1-2* Gene

The physical map of the target gene was constructed via bioinformatic analysis using BAC and P1-derived artificial chromosome (PAC) clones of the cultivar Nipponbare released by the International Rice Genome Sequencing project (IRGSP, http://rgp.dna.affrc.go.jp/IRGSP/index.html). The clones were anchored with the target gene-linked markers, and then, alignment of the sequences was carried out using the pairwise Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/b12.html).

Bioinformatic Analysis

Candidate genes were predicted according to the available sequence annotation information within databases (http://rice.plantbiology.msu.edu/; http://www.tigr.org/). DNA and amino acid sequences were used for a complete alignment using ClustalX version 1.81. The 3-D structures of the TT1-2 protein were predicted and analyzed according to information in a database (https://swissmodel.expasy.org/). The information used for the phylogenetic tree of the *TT1-2* gene was based on available sequence annotation information in a database (http://www.plant.osakafu-u.ac.jp/~kagiana/gcorn/p/).

Results

Evaluation of the Heat Tolerance of Y1502 Under Laboratory Conditions

To evaluate the heat tolerance of Y1502 in the laboratory, a heat tolerance experiment was carried out involving Y1502 and Nipponbare. We found that Y1502 (*Oryza sativa* ssp. *indica*) was much more heat tolerant than Nipponbare (*Oryza sativa* ssp. *japonica*) (see Fig. 1); the survival rates of Y1502 and Nipponbare were 100 and 0, respectively.

Genetic Analysis of the Heat Tolerance Gene

To map the heat tolerance gene in Y1502, the resistant donor Y1502 was crossed with Nipponbare. All the F_1 individuals exhibited a heat-tolerant phenotype. The F_2 individuals were used to analyze the inheritance of the heat tolerance gene in Y1502. The segregation of the heat-tolerant and non-heat-tolerant progeny in the F_2 population fit a 3:1 ratio (153:45 S, v2 = 0.33; Table 1). The segregation ratio suggested that Y1502 harbors a major dominant heat tolerance gene, which was tentatively designated *TT1-2*.

Fig. 1 Phenotypes of Y1502 (right) and Nipponbare (left). Y1502 was much more heat tolerant than Nipponbare in the heat tolerance conditions

Mapping of the *TT1-2* Gene Using Molecular Markers

To identify which gene caused the heat-tolerant phenotype, the no-heat-tolerance allele was genetically mapped. First, a total of 606 SSR and Indel markers from the rice molecular map were subjected to polymorphism surveys involving Y1502 and Nipponbare (McCouch et al. 2002), of which 315 pairs exhibited polymorphism. Based on these 315 primer pairs, 15 heat-tolerant plants and non-heat-tolerant plants from the $F_{2:3}$ population were used for linkage analysis.

Two of these Indel markers, Indel-3-18 and Indel-3-22, were located in the terminal region on chromosome 3 and were found to be linked to the lack of heat tolerance trait among the 193 $F_{2:3}$ individuals. The genetic distance between Indel-3-18 and Indel-3-22 was 20.2 cM according to recombination frequencies. Thus, *TT1-2* was mapped to a 20.2-cM region flanked by the Indel markers Indel-3-18 and Indel-3-22 on chromosome 3 (see Fig. 2a).

Fine Mapping of the *TT1-2* Gene

To map the *TT1-2* gene to a smaller region, 804 individuals with no heat tolerance were identified from the $F_{2:3}$ population derived from Y1502×Nipponbare (Table 1). Additional maps were constructed using published markers (http://archive.gramene.org/markers/) of the region between Indel-3-18 and Indel-3-22. All recombinants were genotyped using five polymorphic markers. The results showed that the *TT1-2* gene was located between the molecular markers RM6594 and RM6931 on chromosome 3, and the genetic distance between the two markers was 5.9 cM (see Fig. 2b and Table 2).

To map the *TT1-2* gene further, eight polymorphic InDels were selected from 22 new InDels (Table 2). These InDel markers were designed from publicly available rice genome sequences, and the likelihood of detecting polymorphisms between Y1502 and Nipponbare was predicted by comparing sequences from Nipponbare (http://rgp.dna.affrc.go.jp/) and

Table 1 Heat tolerance of Y1502

Crosses	F_1 phenotype	F_{23} population			$\chi^2(3:1)$	*P*
		Heat tolerance	No heat tolerance	Total plants		
Y1502/Nipponbare	Heat tolerance	143	42	185	0.33*	0.5–0.75

*The segregation ratio of normal plants to mutant plants complied with 3:1 at 0.05 significant probability level

indica cultivar 93-11 (http://rice.genomics.org.cn/). First, the BAC clone sequences of *japonica* and *indica* were aligned, primers were then designed using Primer Premier 5.0 based on the polymorphic region between the two rice subspecies, and polymorphic markers were used for gene mapping. All recombinants were genotyped using the eight polymorphic markers. The results showed that the *TT1-2* gene was located between the molecular markers IND3-15 and IND3-18 on chromosome 3, and the physical distance between the two markers was 96.0 kb (see Fig. 2c and Table 2).

To finely map the *TT1-2* gene, five polymorphic InDels were selected from 18 new InDels (Table 2). All the recombinants were genotyped using five polymorphic markers. Recombinant screening with seven markers (IND3-15, IND3-29, IND3-31, IND3-34, IND3-37, IND3-40, and IND3-18), which were very close to the *TT1-2* locus, revealed seven, four, two, one, one, three, and three recombinants, respectively. Thus, the *TT1-2* gene was precisely mapped to a 26.0-kb region by IND3-34 and IND3-37 (see Fig. 2d).

Candidate Genes in the 26.0-kb Region

There were three candidate genes (LOC_Os03g26960, LOC_Os03g26970, and LOC_Os03g26980) in the 26.0-kb region (see Fig. 3d) according to the available sequence annotation databases (http://rice.plantbiology.msu.edu/; http://www.tigr.org/): LOC_Os03g26960, which encodes an intron-binding protein; LOC_Os03g26970, which encodes an α2 subunit of the 26S proteasome; and LOC_Os05g02400, which encodes an unknown expressed protein.

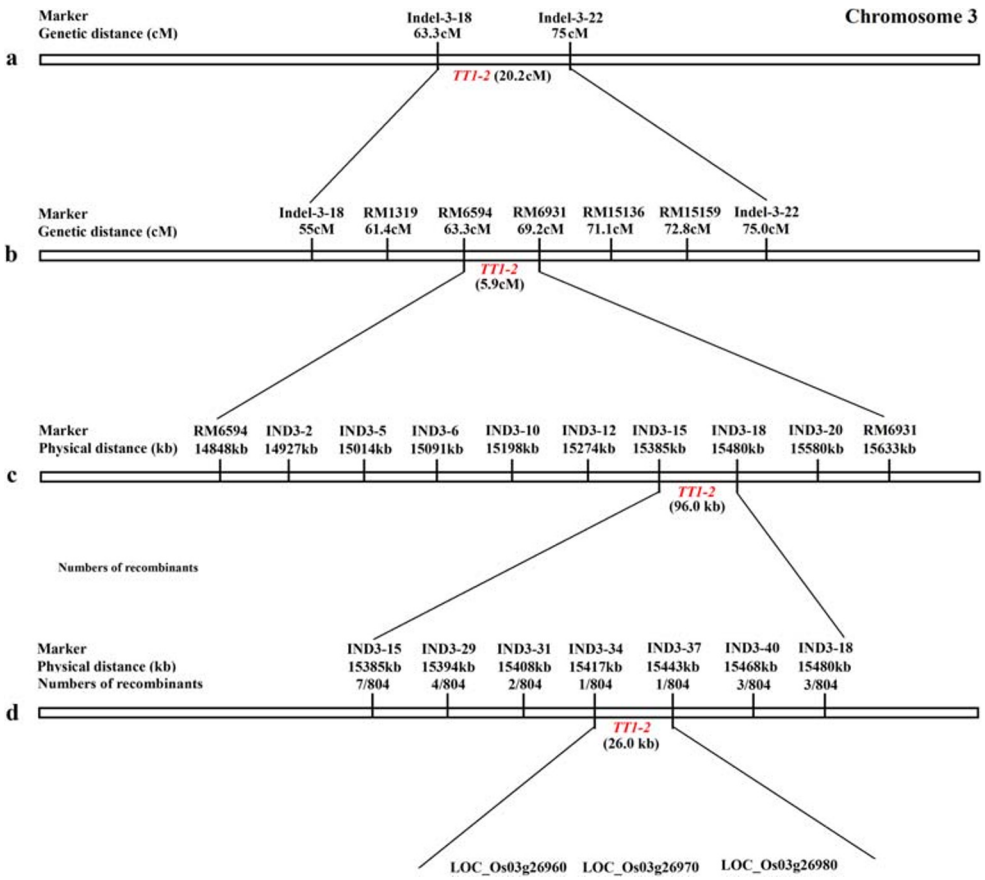

Fig. 2 Genetic and physical maps of the *TT1-2* gene. (a) Primary map of the *TT1-2* gene. The gene was mapped to the region between markers Indel-3-18 and Indel-3-22. (b) Further mapping of the *TT1-2* gene. The gene was mapped to the region between markers RM6594 and RM6931. (c) Further mapping of the *TT1-2* gene. The gene was mapped to the region between markers IND3-15 and IND3-18. (d) High-resolution map of the *TT1-2* gene. The *TT1-2* gene was ultimately localized to a 26.0-kb region between markers IND3-34 and IND3-37, and the recombinant number between the markers and target gene is indicated under the linkage map

Table 2 Indel and SSR molecular markers used for fine mapping of the *TT1-2* gene

Marker	Sequence of forward primer	Sequence of reverse primer
RM1319	CGACGCTGTTGATCCTGTTA	GAAATTAAGCAGCGGAAGCA
RM6594	CTCATGACCTCGTCCTCCTC	ACGTGGTAGCACTCCAGCTC
RM6931	AGTATCATGTAGTGCCACAA	CTCCACTGTAACTCGTTCTC
RM15136	CGAGTTCAGTCTTGGAACCATCC	TGAGCAGTGGAAGACAGTTAGGG
RM15159	CGCTTCCCGAGCTTCTAAAT TATTGC	CGATGTCGCCCAACAATTCTACC
IND3-2	TTTGAGAGTGCCATTTTTCT	ATCTGAACTGAACTGGATCG
IND3-5	AACTGAAACATTTGACCGAC	ATCCAACCAAAATCCCTTAT
IND3-6	CTGACATGTCTCGTTGTTTC	GAGATCACCCTAACATTGGA
IND3-10	AAATGGGTGCAATTTGACT	GATAAAGGGGAGAGAGAGGA
IND3-12	TGACCTGCTTTAGAGTAACC	AACGTTGTTTCTCCTTTTGA
IND3-15	ATAGTTTGAAAAGCGTGTGC	AGTTATGTAAACTCAGGGCA
IND3-18	TCTTGCTCTCATTTGTTCCT	AAATCCTGATGTCCACAAAT
IND3-20	AAGTGCAGTAGTGGTTGTCC	TTATGGTCATCTACCCATCC
IND3-29	CTAATTGGGGACACTATGGA	CTGAATGATGCTGACTGCTA
IND3-31	TGGATACACAGTTGTCCAGA	AATGATTGCTAGCTTTACGC
IND3-34	TAGTCCTGGTTGGTGATGTCTCTGG	CACAATGTGCACCGTGTAGA CAGC
IND3-37	AATGGAGCACTGTAGGTCTGTAGC	CTCACATGTCGAGCTCTATTGC
IND3-40	GGAGGTACCATGAAGTACCA	TTGAGAGCAATTCTACGGTT

IND represents Indel molecular marker, and RM represents SSR molecular marker

Sequence Analyses of the *TT1-2* Gene

To investigate which gene was responsible for the phenotype, sequencing of eight genes in Y1502 and Nipponbare revealed that only one-bp substitution (A-G) was found in LOC_Os03g26970 between Y1502 and Nipponbare (see Fig. 3); no differences in the remaining two gene sequences between Nipponbare and Y1502 were observed. Interestingly, the *OsTT1* (*TT1*) gene, which encodes an α2 subunit of the 26S proteasome, was at this locus (Li et al. 2015). According to the phenotypic resemblance and mapping analysis, we suspected that the heat-tolerant phenotype of *TT1-2* was caused by functional changes at the LOC_Os03g26970 locus. These results indicated that the *TT1-2* gene was probably allelic to *TT1*.

Further analysis showed that the amino acid encoded by the *TT1-2* gene had one amino acid difference; arginine (R)-99 was changed to histidine (H) in LOC_Os03g26970 (see Fig. 4). Although both arginine and histidine are basic amino acids, this change would be expected to alter the function of the protein significantly.

Analyses of the Differences in the 3-D Structure of the TT1-2 Protein

By simulating the spatial structure of the protein, we determined that there was structural difference between the TT1-2 protein and the LOC_Os03g26970-encoded protein (see Fig. 5) Specifically, the 99th amino acid of the LOC_Os03g26970-encoded protein was for arginine (R) instead of histidine (H), which significantly altered the secondary structure (see Fig. 5). Further analysis showed that the TT1-2 protein was able to form a normal alpha-helix (see Fig. 5b, d), while the LOC_Os03g26970-encoded protein was unable to form a normal alpha-helix (see Fig. 5a, c), which may lead to substantial differences in function between the TT1-2 protein and the LOC_Os03g26970-encoded protein.

Phylogenetic Tree of the *TT1-2* Gene

To gain insight into the function of *TT1-2*, a phylogenetic tree was generated using the 26S proteasome protein sequences from rice and other plant species. The phylogenetic tree showed highly homologous genes of *TT1-2* in more than 20 different species (see Fig. 6), and all these genes encoded the same 26S proteasome protein (Table 3). These results indicated that the *TT1-2* gene was highly homologous and conserved across different plant species.

Application of the *TT1-2* Gene in Heat Tolerance Breeding

Utilization of favorable genes has been a priority in rice molecular breeding. Five markers (IND3-29, IND3-31, IND3-34,

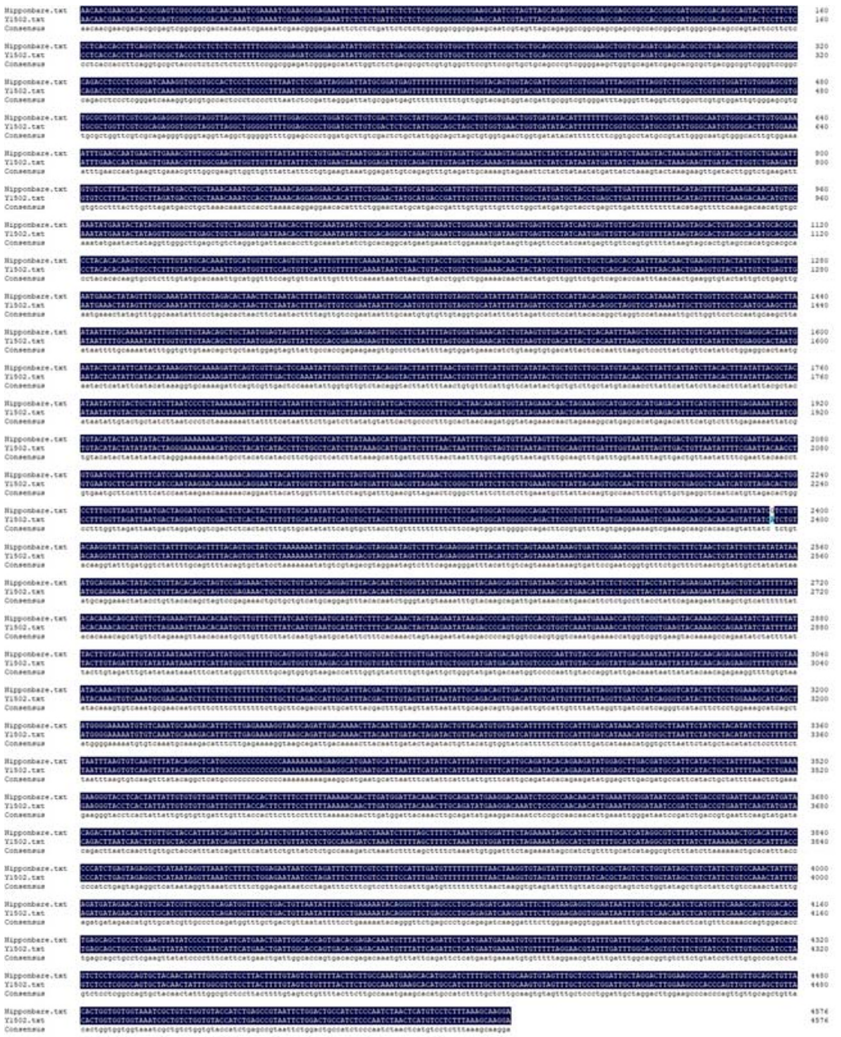

Fig. 3 Sequence comparison of LOC_Os03g26970 between Nipponbare and Y1502, and only one-bp substitution (A-G) was found in LOC_Os03g26970 between Y1502 and Nipponbare

IND3-37, and IND3-40) that were closely linked to the *TT1-2* gene were selected for polymorphism surveys between Y1502 and Xiushui 03, and only IND3-29 exhibited polymorphism. Using marker-assisted selection and conserved breeding selection, we transferred the *TT1-2* gene into a Xiushui 03 maintainer line and developed a new male-sterile line named Zhehang 10A, which showed a high outcrossing rate, good quality, and strong heat tolerance (Table 4); in addition, this new line passed accreditation in Anhui Province in 2019.

Using conventional hybridization techniques, we used three sterile lines of Zhehang 10A as the female parent and the restorer Fuhui 1586 as the male parent and bred a new hybrid—Zhehangyou 1586. Zhehangyou 1586 was grown in Hefei city, Fexi county, Anhui province, in 2019 and showed high yield, good quality, and strong heat tolerance (see Fig. 7).

Discussion

TT1-2 Regulates the Heat Tolerance of Rice

Global warming has the potential to dramatically reduce agricultural harvests, resulting in a widespread risk of food

Fig. 4 Sequence comparison of the amino acid encoded by LOC_Os03g26970 between Nipponbare and Y1502, and the amino acid of the *TT1-2* gene had one amino acid change (99): arginine (R) changes into histidine (H) in LOC_Os03g26970

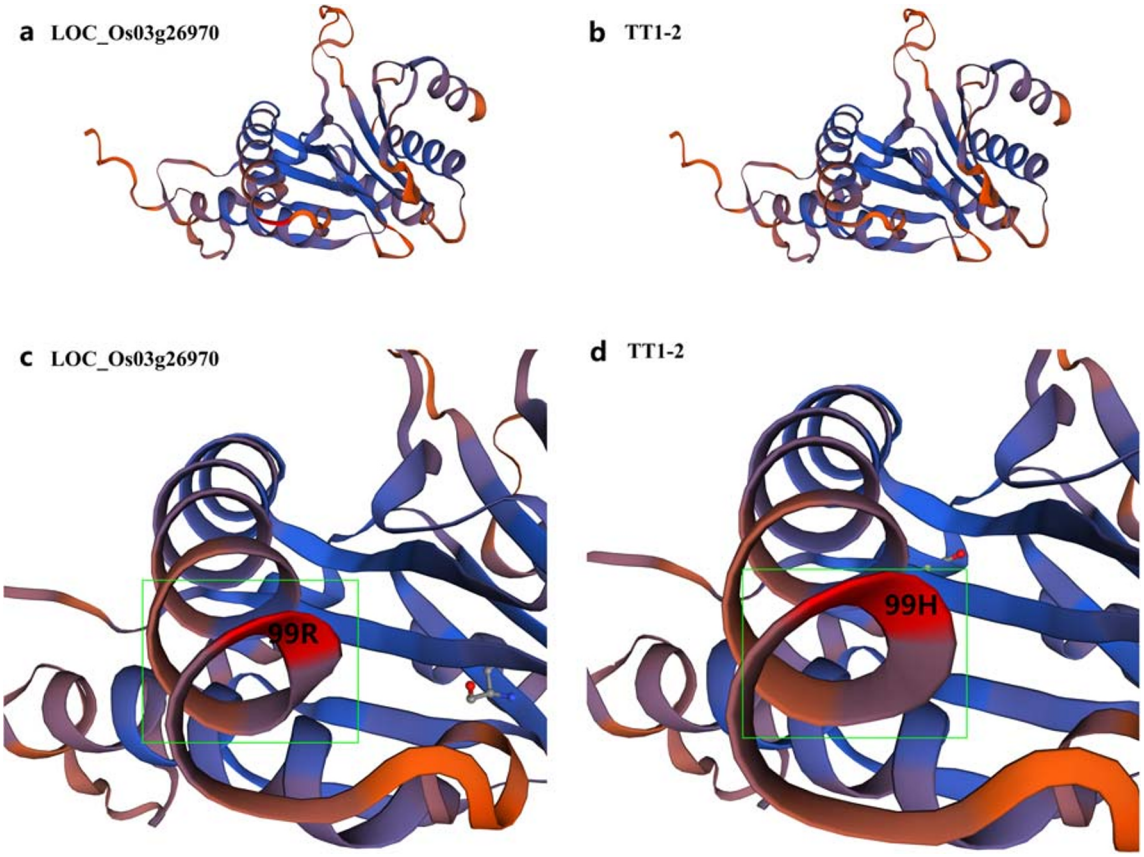

Fig. 5 3-D structures of the protein. **b** and **d** show the protein structure of the TT1-2 protein; **a** and **c** show the protein structure of the LOC_Os03g26970-encoded protein. The green square is the change site of the amino acid

insecurity and social problems (Takeda and Matsuoka 2008). Heat stress leads to severe crop yield losses and reduced milling quality (Lobell et al. 2011; Lyman et al. 2013) and is predicted to result in food crises in the future (Trnka et al. 2014; Semenov and Shewry 2011). Although several heat tolerance genes have been reported in rice, such as *OsSUT1* (Miyazaki et al. 2013), *OsTT1* (Li et al. 2015), *OsHTAS* (Liu et al. 2016), *DPB3-1* (Sato et al. 2016), *qPSL(ht)4.1*, *qPSL(ht)7*, *qPSL(ht)10.2* (Zhao et al. 2016), and *qSTIPSS9.1* (Ps et al. 2017), only one major heat resistance-related gene, *OsTT1*, has been identified. Studies have shown that, compared with *OsTT1*, *OgTT1* protects cells from heat stress through more efficient elimination of cytotoxic denatured proteins and more effective maintenance of heat-response processes and plays an important role in local adaptation during rice evolution (Li et al. 2015). In this study, a rice heat tolerance gene, *TT1-2*, was isolated by map-based cloning, and the *OsTT1* gene was present at this locus (Li et al. 2015), which indicated that the *TT1-2* gene was probably allelic to *TT1*. Moreover, the *TT1-2* gene was a dominant gene, which serves as a valuable genetic resource for breeding heat-tolerant plants.

Molecular Functional Analysis of the *TT1-2* Gene

The 3-D structures of *TT1-2* and LOC_Os03g26970 based on the simulation showed that the exchange of histidine (H) for arginine (R) alters the protein structure, which resulted in a failure to form a normal alpha-helix (Fig. 5). Studies have shown that the BnRCH protein can significantly improve plant heat resistance, while histidine conversion and cysteine play important roles in the function and classification of the BnRCH protein (Sakamoto et al. 2004). We suspected that histidine may play an important role in maintaining protein function in our case. Studies have shown that heat-resistant varieties have higher yields, seed setting rate, and spikelet numbers; the reason was that the heat tolerance of rice was related to concentrations of phytohormones such as GA3, IAA, and ABA in rice spikelets (Zhang et al. 2017; Khan et al. 2019). Researchers isolated a *cytokinin-receptor kinase* (*CER1*) from plants and inferred that histidine kinase was involved in the transmission of cytokinin signals (Inoue et al. 2001). We speculate that the *TT1-2* gene may enhance the heat tolerance of rice by regulating cytokinin levels.

Table 3 Homolog gene of the *TT1-2* gene by species

Species	Repr. RefSeq ID	Annotation
Zea mays	NP_001132248	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Sorghum bicolor	XP_002452496	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681184	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681185	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681186	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681187	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681188	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681189	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008681190	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Setaria italica	XP_004953204	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008676492	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008678850	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	XP_008678852	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Zea mays	NP_001288516	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Setaria italica	XP_004984179	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Sorghum bicolor	XP_002467767	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Oryza brachyantha	XP_006648864	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Oryza sativa japonica group	XP_015625377	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Brachypodium distachyon	XP_003575428	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Oryza sativa japonica group	XP_015628148	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.
Oryza brachyantha	XP_015690380	Proteasome subunit alpha type; proteasome subunit alpha; Provisional; proteasome_alpha_type_2. The 20S proteasome, multisubunit proteolytic complex, is the central enzyme.

Utilization of the *TT1-2* Gene in Rice Breeding

Plant breeding, which aims to improve the genetic basis of new varieties of crops with increased productivity and quality, combines art and science (Xi et al. 2006). In general, traditional breeding is predominantly based on phenotypic assays (Xu et al. 2010), but this approach targets only traits with an easily observable phenotype for genetic improvement. Moreover, heat tolerance is an unobservable trait that requires further phenotypic identification. However, we can improve the heat resistance of rice by molecular marker-assisted selection.

Xiushui 03 is a maintainer line of hybrid rice, and Zhehang 10A is a cytoplasmic male-sterile line of hybrid rice. In this paper, we transferred the *TT1-2* gene into the maintainer line Xiushui 03 using the molecular

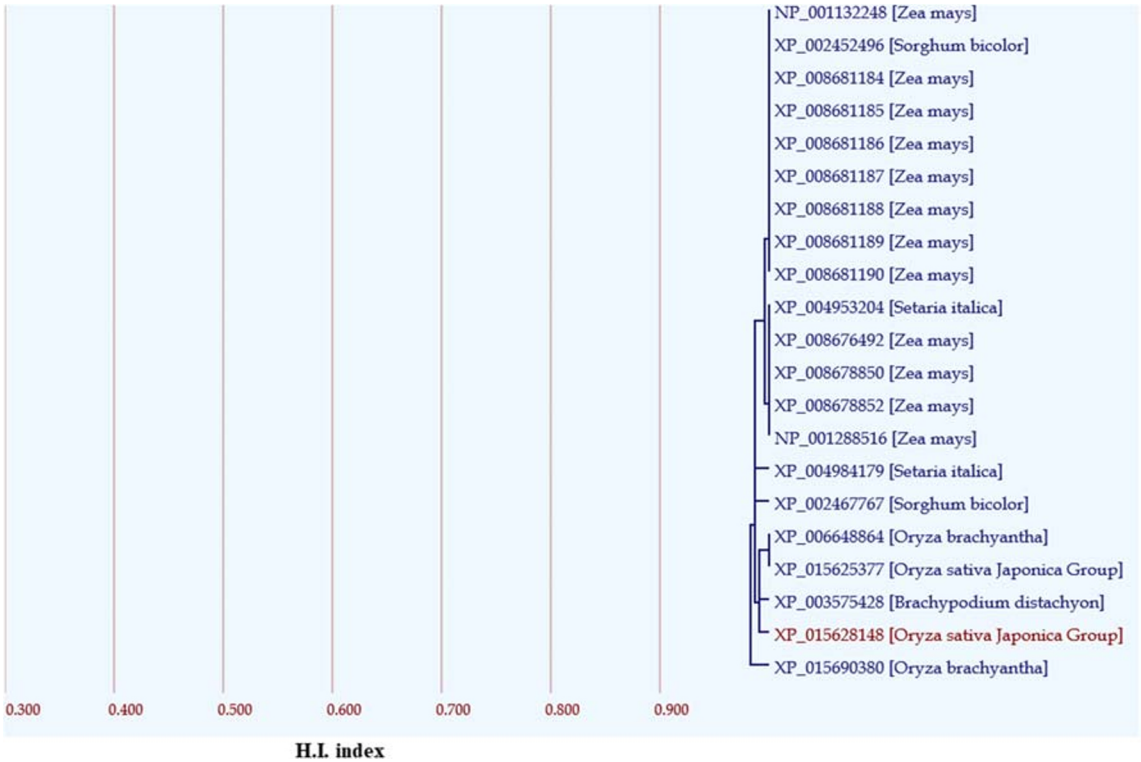

Fig. 6 Phylogenetic tree of the *TT1-2* gene. The *X* axis of the phylogenetic tree represents the H.I. index (0.300 to 1.000), and 0.3 means the ratio of similar amino acids between the two genes is 30%

marker IND3-29 and developed a new male-sterile line, Zhehang 10A, which showed a high outcrossing rate, good quality, and strong heat tolerance. Moreover, the *TT1-2* gene was controlled by a dominant gene (Table 1). Therefore, to breed new hybrid rice varieties, breeders can transfer this gene into either restorer or sterile lines using marker-assisted selection. For example, we took Zhehang 10A (which contains *TT1-2*) as the female parent and the restorer Fuhui 1586 (which lacks *TT1-2*) as the male parent and bred a new hybrid—Zhehangyou 1586. Compared with Tianxie 1 (CK), Zhehangyou 1586 showed higher yield, had better quality, and presented stronger heat tolerance (Table 4). Therefore, the *TT1-2* gene has good application prospects in future genetic breeding.

Table 4 Comparison of the main agronomic traits between Zhehangyou 1586 and Tianxie1hao

Traits	Zhehangyou 1586	Tianxie1hao(CK)
Plant height (cm)	118.62	120 .22
Number of effective panicle	10.06	9.82
Spikelets per panicle	245.30**	182.36**
Seed setting rate (%)	89.72*	84.34*
1000-grain weight (g)	25.64	26.52
Yield per plant (g)	56.76**	44.58**
Brown rice rate (%)	81.24	80.68
Head rice rate (%)	69.52*	61.13*
Tolerance	Heat tolerance	No heat tolerance

*Difference between Zhehangyou 1586 and Tianxie1hao at $P < 0.05$; **at $P < 0.01$. Data are derived from the trial performed at Fexi county of Anhui province in October 2019

Fig. 7 Field performance of the new rice variety Zhehangyou 1586

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Author Contribution CY drafted the manuscript. CY, DY, GZ and HX contributed to the data analysis. DY and CY participated in the design of the study and the interpretation of the results and wrote and edited the manuscript.

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Compliance with Ethical Standards

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

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