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The analyses of mapping tightly linked markers of a single dominant heat-tolerance gene in indica variety, TCS17

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ABSTRACT

In the past three decades, global warming has led to an increase in annual average temperature. This high temperature stress is a serious threat to the growth and yield in rice. Therefore, scientists study hard to find heat-tolerance genes and breed heat-tolerance rice lines. Ten years ago, a 100%-explaintion heat-tolerance gene was discovered in my salt-tolerance research on screening, inheritance and linkage marker analyses of salt tolerance in mutated scented japonica rice, to develop elite salt-tolerant varieties suitable for salinized areas[24,25]. In view of this, I began the first analysis of mapping tightly linked markers of a single dominant heat-tolerance gene in indica variety, TCS17, explaining 100% of the phenotypic variation. I reported the results of this heat-tolerance gene analysis, hoping to help scientists to find salt-tolerant genes and cultivate salt-tolerant strains. The analyses can solve the problem of high temperature stress in rice and help humans deal with future food shortages. Yen (2011) and Yen and Lin (2011) treated the seedlings of 1005 mutated scented japonica rice lines with six true leaves with 300 mM NaCl for three days. Only the salt-tolerant line, SM61, survived. We obtained F_2 populations from the cross between a salt-tolerant and heat-susceptible japonica line, SM61, and a salt-susceptible and heat-tolerant indica variety, TCS17. After culture with 35/25°C (day/night) and 200 mM NaCl for five days, the heat-tolerant and salt-tolerant surviving (R) to heat-sensitive or salt-sensitive non-surviving (S) ratio in 290 F_2 plants showed a good fit to the Mendelian 3 : 13 segregation ratio by a Chi-square test indicating that the heat-tolerance of TCS17 was governed by a single dominant gene. The heat-tolerance gene explained close to 100% of the total phenotypic variation, and was tightly linked to C61009 (marker) located on chromosome 4. This is the first report of mapping tightly linked markers of a single dominant heat-tolerance gene. These linkage markers can efficiently provide the fine mapping, cloning and sequence comparison, as well as the functions of the new heat-tolerance gene, answering the question of how the gene controls heat tolerance.

KEYWORDS

Inheritance; Mutated; *Oryza sativa*; Quantitative trait loci (QTLs); Salt tolerance; Heat tolerance; Scented; Seedling stage.

INTRODUCTION

Rice feeds more than half of the world 's population. However, in the increase of average temperature due to global warming, rice is suffering from high temperature stress, resulting in threats to the decrese of the growth and yield in rice. Next to drought and salinity stress, heat stress is the third abiotic threat to rice production and quality. Therefore, scientists around the world are actively searching for heat-tolerance genes and breeding heat-tolerance rice strains[1,5,15]. In previous studies[7,13,15,21-23,26,27], the location of QTLs for heat

tolerance in rice was examined by using molecular markers. Zhu et al. (2005) found three QTLs involved in controlling heat tolerance of rice during grain filling on chromosomes 1, 4 and 7. Ye et al. (2011) mapped two major heat tolerance QTLs, qHTSF1.1 and qHTSF4.1 in relation to spikelet fertility at the flowering stage on chromosomes 1 and 4. Ye et al. (2015) and Ye et al. (2015) performed the identifying, confirming, fine-mapping and validating of qHTSF4.1 to increase spikelet fertility under heat stress at flowering in rice. Zhao et al. (2016) found eleven related QTLs were detected, two QTLs for spikelet fertility on chromosomes 2 and 4, four QTLs for daily flowering time on chromosomes 3, 8, 10 and 11, and the other five for spikelet fertility and pollen shedding level on chromosomes 1, 4, 5, 7 and 10, respectively. Shanmugavadivel et al. (2017) identified five QTLs involved in stress susceptibility and stress tolerance indices (SSI and STI) of percent spikelet sterility and yield per plant on chromosomes 3, 5, 9 and 12. Kilasi et al. (2018) identified fifteen related QTLs were detected, one QTL for root length under heat stress on chromosome 5, four QTLs for root length under heat stress as percent of control on chromosomes 1, 2, 3 and 4, three QTLs for shoot length under heat stress on chromosomes 3, 4 and 6, and the other seven for shoot length under heat stress expressed as percentage of control on chromosomes 2, 3, 4, 5, 6 and 10, respectively. Nubankoh et al. (2020) detected QTLs for spikelet fertility under heat stress on chromosomes 1, 2 and 3 in rice. Most QTLs related to heat-tolerance were located in a similar region on chromosome 4[7,21-23,26,27]. Identification of new QTLs related to heat tolerance through marker-assisted selection (MAS) should be useful in rice heat-tolerant breeding programs[7,13,15,21-23,26,27].

The purpose of this study was to analyze linkage markers and heritage characters of the indica variety, TCS17, for a single dominant heat-tolerance gene by using F_2 populations derived from a cross between a heat-tolerant and salt-susceptible indica variety, TCS17, and a heat-susceptible and salt-tolerant japonica line, SM61. The results will help begin map-based cloning of the single dominant heat-tolerance gene and elucidate the mechanism controlling the heat-tolerance gene, and will be useful in breeding programs of heat tolerance in rice.

MATERIALS AND METHODS

Plant materials

Yen (2011) and Yen and Lin (2011) performed the experiments on screening of salt tolerance in 1005 mutated scented japonica lines originated from the wild-type japonica cultivar, TNG67[4]. TNG67 was treated with a chemical mutagen, NaN_3, to produce the steady genetic line of mutated scented rice, CNY911303. Then, CNY911303 was treated with EMS to produce the second generation of 1005 mutated lines that developed to the sixth generation with superfine agronomic traits in the first season of 2006[20]. These 1005 lines, at the three true leaf growth stage, growing in the National Chung Hsing University glasshouse were treated with 150 mM NaCl for three weeks. According to the procedure of Verma et al. (2007), 11 salt-tolerant lines were selected, grown and developed to the seventh generation. All 13 lines, including the 11 selected lines, CNY911303, and TNG67 seedlings with six true leaves, were treated with 300 mM NaCl for three days at a mean temperature of 30/20°C (day/night)[14]. SM61, was the only surviving line which produced seeds. This salt-tolerant and heat-susceptible japonica line, SM61, was hybridized with a salt-susceptible and heat-tolerant indica variety, TCS17, and F_1 seeds were produced in the second season of 2007[8,10,24,25]. The F_1 plants were self-fertilized to develop the F_2 seeds in the first season of 2008.

Linkage analysis of heat-tolerant plants

Yen (2011) and Yen and Lin (2011) performed the experiments on linkage analysis, 290 F_2 plants (SM61×TCS17) were cultured in a hydroponic culture medium in a plastic tray (60 × 48 × 16 cm) under non-shaded conditions at National Taiwan University during July and August in 2008. The seedlings were cultured at a mean temperature of 35/25°C (day/night). At the four true leaf stage, the F_2 plants were cut at about the 5 cm leaf level and DNA was extracted. The plants with six true leaves were grown in a hydroponic culture medium supplemented with 200 mM NaCl for five days. After the five-day culture, the 290 F_2 plants were

transferred to the hydroponic culture medium without NaCl. 52 F_2 heat-salt-tolerant surviving plants were selected. TNG67 was heat-susceptible and salt-susceptible, SM61 was heat-susceptible and salt-tolerant, while TCS17 was heat-tolerant and salt-susceptible[8,10,24,25]. Among the 52 F_2 heat-salt-tolerant surviving plants, 46 plants were screened for constructing the linkage markers map of the salt-tolerant gene in SM61 and the heat-tolerant gene in TCS17.

The 46 selected F_2 heat-salt-tolerant plants were used to construct a linkage map. The primer sequence designs of the SSR marker, STS marker and Indel marker, belonging to PCR markers, were carried out on the basis of the information offered by Harushima et al. (1998), Lin et al. (1998), Temnykh et al. (2001), McCouch et al. (2002), Shen et al. (2004), Lin et al. (2008). In total, 106 SSR markers, STS markers and Indel markers were discovered. All markers covered the entire rice genome, which revealed polymorphisms between the parents, SM61 and TCS17, which are represented by A and B, respectively, and were used to determine the genotypes of the selected F_2 heat-salt-tolerant plants. The map spanning between the two markers was around 20 centiMorgans (cM). We used Microsoft Excel 2000 software to calculate the genotypic frequency distributions of the F_2 heat-salt-tolerant plants.

Inheritance analysis of heat tolerance

To confirm that the gene controlling heat-tolerance in TCS17 was a single dominant gene, I performed the inheritance analysis. In Yen (2011) and Yen and Lin (2011)'s experiments on linkage analysis, the 290 F_2 plants (SM61×TCS17) were grown in a hydroponic culture medium supplemented with 200 mM NaCl at a mean temperature of 35/25°C (day/night) for five days (60 × 48 × 16 cm container). TCS17 was heat-tolerant and salt-susceptible while SM61 was heat-susceptible and salt-tolerant[8,10,24,25]. After the five-day culture, the 290 F_2 plants were transferred to the hydroponic culture medium without NaCl. After one week, their heat-tolerant and salt-tolerant surviving (R) and heat-sensitive or salt-sensitive non-surviving (S) frequency distribution was calculated. The R to S ratio of F_2 populations were analyzed to fit a Mendelian 1 : 3 or 3 : 13 segregation ratio using a Chi-square test from SAS software.

RESULTS AND DISCUSSION

Results

Linkage analysis of a heat-tolerance gene

In Yen (2011) and Yen and Lin (2011)'s experiments on linkage analysis, the 290 F_2 (SM61×TCS17) plants were treated with 200 mM NaCl at a mean temperature of 35/25°C (day/night) for five days under non-shaded conditions at National Taiwan University during July and August in 2008. After the five-day culture, the 290 F_2 plants were transferred to the hydroponic culture medium without NaCl. The 52 salt-tolerant and heat-tolerant plants developed new leaves and roots quickly while the salt-sensitive or heat-sensitive plants did not survive. We screened 46 of the 52 F_2 heat-salt-tolerant surviving plants to map the salt-tolerant gene in SM61 and the heat-tolerant gene in TCS17. The genotypes of TNG67, TCS17, and heterozygote are represented by A, B, and H, respectively. The A, H and B genotypic frequency of the C61009 marker (8.7 cM) was 0, 20, and 26, respectively (TABLE 1) (Figure 1). I predicted that, the H and B genotypic percentage of the C61009 marker occupied 100 % of chromosome 4, the heat-tolerance of TCS17 was governed by a single dominant gene. Based on the linkage correlation between the heat-tolerant marker genotypes and phenotypes, the phenotypic variance in the F_2 plants was explained by the C61009 marker on chromosome 4. Since 290 F_2 rice seedlings were treated with 200 mM NaCl for five days at a mean temperature of 35/25°C (day/night), the heat-tolerance gene explained close to 100% of the total phenotypic variation, and was tightly linked to C61009 (marker) located on chromosome 4. Thus, the heat-tolerance gene, *HT4*, which conferred the heat tolerance of TCS17 was mapped on chromosome 4 flanked C61009 marker.

Inheritance analysis of heat tolerance in TCS17

To exclude heat stress, in Yen (2011) and Yen and Lin (2011)'s experiments on inheritance, with 200 mM NaCl in a net-house at a mean temperature of 30/20°C (day/night) for five days, SM61 and F_1 (SM61×TCS17; TCS17×SM61) plants survived (R) while TCS17 plants did not (S). The R to S ratio in 513 F_2 plants showed a good fit to the Mendelian 3 : 1 segregation ratio by a Chi-square test indicating that the salt-tolerance of

SM61 was governed by a single dominant gene.

Since Yen (2011) and Yen and Lin (2011) indicated that the salt-tolerance of SM61 was governed by a single dominant gene, we needed to use salt-sensitive non-survivals to map the linkage markers of the salt-tolerance gene in SM61. However, in Yen (2011) and Yen and Lin (2011)'s experiments on linkage analysis, the 290 F_2 plants were cultured under non-shaded conditions at a high mean temperature of 35/25°C (day/night) during July and August. The screened environment under the salt-stress and heat-stress conditions at National Taiwan University in the hottest season produced a majority of dead plants preventing the selection of salt-sensitive non-survivals for mapping. Therefore, the only 52 heat-salt-tolerant F_2 plants survived. Among the 52 F_2 surviving plants, we selected 46 survival plants to perform the mapping. The performance in each the F_2 heat-salt-tolerant plants proved that the genotypes of the 46 salt-tolerant F_2 plants were homozygote dominant (A), not heterozygote dominant (H). The number of genotype A of the RM223 marker (75.7 cM) in the 46 F_2 salt-tolerant surviving plants were 46 (100%) (TABLE 1). We predicted that, since the salt-tolerance in the 46 F_2 plants was governed by a homozygote dominant allele, the salt-tolerance gene in SM61 might be semidominant.

Among 290 F_2 plants grown in the culture medium without NaCl for one week, the number of R and S plants were 52 and 238, respectively. The heat-tolerance gene, *HT4*, which conferred the heat tolerance of TCS17 was mapped on chromosome 4 flanked C61009 marker; the salt-tolerance gene, *ST8*, which conferred the salt tolerance of SM61 was mapped on chromosome 8 flanked RM223 marker. These two genes were located on diffreent chromosomes. The R to S ratios of the F_2 population fitted a Mendelian 3 : 13 segregation ratio by the Chi-square test, but didn't fit a 1 : 3 segregation ratio (TABLE 2). The results indicated that R (the 52 heat-salt-tolerant F_2 survival rice plants) was governed by a single dominant heat-tolerant gene and a homozygote-semidominant salt-tolerant allele, not only governed by a homozygote-semidominant salt-tolerant allele. Morever, the A, H and B genotypic frequency of the C61009 marker (8.7 cM) was 0, 20, and 26, respectively. The H and B genotypic percentage of the C61009 marker occupied 100 % of chromosome 4 (TABLE 1) (Figure 1). I predicted that, since the inheritance and linkage marker analyses of heat tolerance in TCS17, the heat-tolerance of TCS17 was governed by a single dominant gene.

Discussion

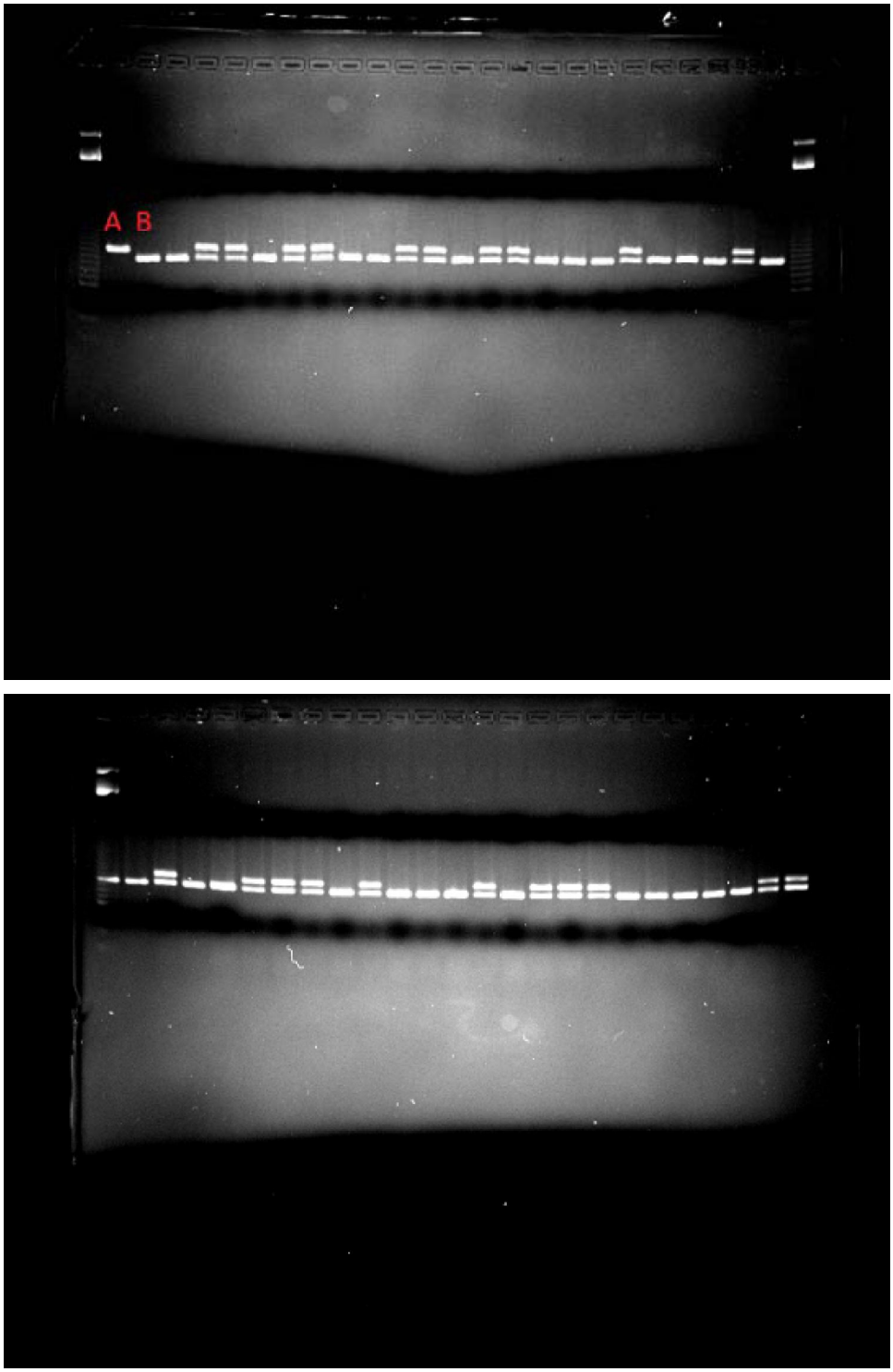
Since during salt or drought screening in rice, high heat stress has to be excluded during dry and hot season, either heat sensitivity directly or raising tissue temperature by reducing transpiration under water deficit conditions indirectly[1,6,24,25]. Furthermore, the main mechanisms of salt tolerance in crops include: ion homeostasis, osmotic homeostasis, and control and repair of oxidative stress damage[19]. The high temperature tolerant indica variety, TCS17 tends to be more heat-tolerant than a high temperature sensitive japonica cultivar, TNG67[8,10]. Morever, in Yen (2011) and Yen and Lin (2011)'s experiments on screening and inheritance of salt tolerance, the 1005 mutated scented japonica rice lines[20] were originated from the wild-type japonica cultivar, TNG67[4]. Therefore, to exclude high heat stress, we cultured the lines in a net-house at a mean temperature of 30/20°C (day/night). However, in Yen (2011) and Yen and Lin (2011)'s experiments on linkage analysis, the 290 F_2 plants were cultured with 200 mM NaCl for five days under non-shaded conditions at a high mean temperature of 35/25°C (day/night) during July and August. Under the salt-stress and

TABLE 1: The genotypes of markers in 46 heat-salt-tolerant F_2 plants on chromosome 4 and chromosome 8.

Chr.	Location (cM)	Marker	A	H	B	N
4	8.7	C61009	0	20	26	0
4	28.6	SLS189	5	15	25	1
4	77.9-78.2	RM252	16	16	14	0
4	87.1-94.4	RM303	12	20	14	0
4	108.2	S13714	10	23	13	0
8	0.5	RM337	10	22	14	0
8	21.6-25.2	SLS182	9	24	8	5
8	45.4	RM72	20	20	6	0
8	54.3	RM331	21	19	6	0
8	75.7	RM223	46	0	0	0
8	92.2	SLS188	15	23	8	0
8	105.7-106.1	CH0862	11	27	8	0

The genotypes of TNG67, TCS17, heterozygote, and non-detection are represented by A, B, H, and N, respectively[24,25].

C61009 (8.7 cM) size: TNG67(A):210; TCS17(B):190



the first row

the second row

Figure 1: The genotypes of C61009 marker on chromosome 4 in the parents, TNG67 (A, the first row, lane 1) and TCS17 (B, the first row, lane 2), and the 46 heat-salt-tolerant F_2 rice plants (lane 3-24 of the first row and lane 1-24 of the second row)

heat-stress treatment, the only 52 salt-heat-tolerant F_2 plants survived. The H and B genotypic percentage of the C61009 marker occupied 100 % of chromosome 4 and the A genotypic percentage of the RM223 marker occupied 100 % of chromosome 8, which on different chromosomes. Chi-square values indicated that the F_2 population had good fits to a Mendelian 3 : 13 segregation ratio for dominant heat-tolerant and

homozygote-dominant salt-tolerant (R) and recessive heat-sensitive or non-homozygote-dominant salt-sensitive (S) traits. These results indicate that the heat-tolerance of TCS17 was governed by a single dominant gene. This is the first report that in F_2 heat-tolerant rice plants, the heat-tolerance is governed by a single dominant gene. By analyzing the inheritance of the single dominant heat-tolerance gene in TCS17, I can confirm the best method for analysis of the linkage markers in TCS17 for the heat-tolerance gene.

I analyzed the selected 46 F_2 heat-salt-tolerant plants to map the linkage markers of the heat-tolerance gene, TCS17. I found that the TCS17 heat-tolerance gene, *HT4*, is located on chromosome 4, different from those positions of previous QTL regions previously discovered on chromosome 4[7,21-23,26,27]. By applying MAS techniques to the gene I have identified as responsible for conferring heat-tolerance, the results of my study could be used to improve heat tolerance in rice. Morever, the heat-tolerance in the 46 F_2 plants was governed by a single dominant gene. Dominance would be helpful in the development of new elite varieties of plant that have a high-level of heat tolerance. Selected heat-tolerant lines like TCS17 and RIL will provide the new breeding sources for the production of heat-tolerant lines with superfine agronomic traits in the field.

TABLE 2: The frequency distribution of salt-tolerant and heat-tolerant survival (R) and salt-sensitive or heat-sensitive non-survival (S) in F_2 population evaluated by Chi-squared values to fit a Mendelian 1 : 3 segregation of a single homozygote-dominant salt-tolerant gene model, or a 3 : 13 segregation of a single homozygote-dominant salt-tolerant gene and a single dominant heat-tolerant gene model. The rice plants at the six true-leaf stage were cultured in hydroponic culture medium containing 200 mM NaCl at a mean temperature of 35/25°C (day/night) for 5 days, and then transferred to a hydroponic culture medium without NaCl and grown for one week.

Rice population	Observed Frequency		Total plants	X^2	P[4)]
	R[1)]	S[2)]			
F_2[3)] $_{1:3}$	52	238	290	7.7287	0.0054
F_2[3)] $_{3:13}$	52	238	290	0.1277	0.7209*

[1)] salt-tolerant and heat-tolerant survival rice plants; [2)] salt-sensitive or heat-sensitive unsurvival rice plants; [3)] 290 F_2 rice plants derived from the cross (SM61×TCS17); [4)] * represents values to fit a 1 : 3 segregation of a single homozygote-dominant salt-tolerant gene model, or 3 : 13 segregation of a single homozygote-dominant salt-tolerant gene and a single dominant heat-tolerant gene model at P>0.05.

Based on the mapping results, I presumed that the different location of the heat-tolerance gene, *HT4*, was obtained in F_2 generations derived from a heat-susceptible and salt-tolerant japonica parent, SM61 and a heat-tolerant and salt-susceptible indica parent, TCS17. The materials in my study were different from those in the other reports investigating RIL and DH lines[7,13,15,21-23,26,27] since the latter derived from a cross between Nagina22, a well-known heat tolerant aus cultivar and IR64, a heat sensitive popular indica rice variety[7,15,21-23] or from a heat-tolerant indica parent, Habataki, and a heat-susceptible japonica parent, Sasanishiki[26] or from a heat-tolerant parent, M9962, and a heat-susceptible parent, Sinlek[13]. To my knowledge, this is the first study using mapping materials originating from a heat-susceptible and salt-tolerant japonica line, and a heat-tolerant and salt-susceptible indica variety. The materials of screened heat-salt-tolerant F_2 plants (SM61×TCS17) resulted in my findings of a single dominant heat-tolerant gene in TCS17 and a homozygote-semidominant salt-tolerant allele in SM61 simultaneously.

The locations of QTLs of heat tolerance in rice have been reported[7,13,15,21-23,26,27], but they did not refer to tightly linked markers for a single dominant heat-tolerance gene. In these reports, QTLs explained less than 30% of the total phenotypic variance for the heat-tolerance[7,13,15,21-23,26,27], and most QTLs accounted for less than 20% of the heat-tolerance. My study was therefore the first to map for tightly linked markers of a single dominant heat-tolerance gene, explaining 100% of the phenotypic variation. Only when these four conditions exist could I have an opportunity to find a explaining 100% heat-tolerant gene[1,2,6]: (1) Under the salt or drought stress, high temperature stress is added, the damage of high temperature stress will reach a high level. Therefore, it can be seen whether the plant is extremely heat-tolerant under the stress of salt or drought treatment with heat stress simultaneously. (2) The heat-tolerance properties of all heat-tolerance studies have been expressed in a continuous distribution (stress susceptibility and stress tolerance indices). Only my study was the survival or death of plants as the judgment of heat tolerance, divided into two parts, expressed in qualitative characters. (3) My research is to cultivate

plants by hydroponic cultivation, different from other heat-tolerant studies on planting with soil. There are many interference factors in the soil, my study could deduct many interference factors in the environment. (4) Indica variety, TCS17, has a 100%-explaintion heat-tolerance gene for regulation and signaling. This heat-tolerance gene is likely to regulate other heat-tolerance genes and initiate the expression of all heat-tolerance traits of mitigating damage for growth, yield, grain quality, spikelet sterility, root length and shoot length under heat stress in rice. It is urgent to directly clone the heat-tolerance gene with a 100% explaintion. On the breeding of heat-tolerant strains, the ideal variety can be crossed with indica variety, TCS17, and the RILs closely linked with C61009 marker can be selected for breeding.

CONCLUSIONS

In response to climate change due to global warming, there is an urgent need to find high-explaination heat-tolerant genes in the world. My study will explain the 100% phenotypic variation of heat tolerance in indica variety, TCS17, which is expected to be provided to global research and fine map of the highly heat-tolerance gene, to bring the fastest breeging strategy of high temperature tolerant rice in the future.

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