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Unraveling the Genetic Architecture for Low Temperature Germinability-Related Traits in Rice Using Genome-Wide Association Study

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Abstract: Rice is frequently affected by cold weather at high altitudes in temperate and subtropical regions. With the popularity of direct seeding, a better understanding of the genetic mechanisms regulating cold tolerance will enable breeders to develop varieties with strong low temperature germinability (LTG). In this study, six indices including low temperature germination percentage (LTGP), relative germination percentage (RGP), relative plumule length (RPL), plumule length after 6-day recovery (PLR), plumule length recovery rate (PLRR) and recovery ability of plumule length after cold stress (RAPL) were measured to assess LTG, and carried out a genome-wide association study (GWAS) to identify QTL and candidate genes related to LTG by using a natural population comprising 211 rice accessions. A total of 18 QTL including two for LTGP, three for RGP, five for PLR, four for PLRR, two for RPL and two for RAPL were uncovered on 12 chromosome regions located in chromosome 1, 2, 4, 5, 6, 9, 10 and 12. On chromosome 2, *qLTGP2* and *qRGP2* were co-localized at 3.3 Mb, and *qPLR2* and *qPLRR2* were co-localized at 5.5 Mb; *qLTGP5*, *qPLR5* and *qPLR5* were co-localized at 27.8 Mb on chromosome 5; *qPLR6* and *qPLRR6* were co-localized at 5.7 Mb on chromosome 6; and *qPLR12* and *qPLRR12* were co-localized at 23.5 Mb on chromosome 12. These results indicated that some LTG-related traits may share the same genetic pathway. For the 18 LTG-related QTL, seven QTL (*qLTGP2*, *qRGP2*, *qPLR2*, *qPLRR2*, *qLTGP5*, *qPLR5* and *qPLR5*) were reported for the first time. According to candidate gene analysis, fourteen genes from five QTL (*qLTGP2*, *qPLR2*, *qLTGP5*, *qRAPL10* and *qPLR12*) were considered as candidate genes and will be further functionally validated in subsequent experiments. QTL with superior candidate genes identified in this study will be useful in improving cold tolerance in rice cultivars. The rice varieties with strong LTG identified in this study will enrich the resources of rice cultivation project.

Keywords: cold tolerance; accessions; GWAS; low temperature germinability (LTG); QTL

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1. Introduction

Rice (*Oryza sativa* L.) is a monocotyledon model plant, which is the food source for more than half of the world's population [1]. Due to the long growing season and frequent low temperatures in high latitudes such as northern China, Korea and Japan, the growing time of low-temperature intolerant rice cultivars must be shortened, which usually results in low yields [2]. In many rice growing countries in Asia, direct seeding has

become an alternative to traditional rice transplanting, because it reduces labor demand and production cost, and its importance and popularity are becoming stronger [3]. However, since the temperature of sowing in these areas is often below 15 °C, direct sowing usually results in seedling poor establishment due to low germination rates at low temperatures. Therefore, the LTG of rice has become a crucial factor to determine whether direct seeding rice can thrive. Improvement of LTG allows for high germination vigor and stable seedling establishment under low-temperature production environments, which leads to yield stability, because improved cold tolerance during germination will allow direct seeding rice to be planted earlier in the season, allowing rice crops to take advantage of the usually abundant rainfall early in the growing season [4,5].

The LTG of rice is a very complex trait. Bi-parental mapping studies have identified more than 100 QTL distributed on all 12 chromosomes associated with LTG [6]. Among these QTL, some of them have been fine mapped. For example, *qLTG-9* was fine mapped to a 72.3 kb physical region on chromosome 9 [7], *qSV-5c* was located in a genomic region of approximately 400 kb on chromosome 1 [8] and *qLTG6* was delimited to a 45.8 kb physical region on chromosome 6 [9]. *qLTG3-1* was the first gene identified to associate with LTG, codes for a secreted hybrid glycine-rich protein, and a single nucleotide substitution differentiates between strong and weak alleles and strongly expressed in the embryo during seed germination [10,11]. However, most of the favorable alleles of the QTL associated with LTG that have been published all derived from *japonica* rice in bi-parental populations, the major drawback of bi-parental mapping is the limitation of genetic diversity. Moreover, all of these LTG-related QTL were identified using traditional markers, making it difficult to obtain complete, precise positional information about these LTG related QTL [12].

Single nucleotide polymorphisms (SNPs) have been widely used in GWAS instead of traditional SSR markers; therefore, GWAS has become a new strategy to detect QTL and genes related to target traits. In recent years, researchers have made some progress in using GWAS to mine QTL associated with LTG, e.g., 17 LTG-related QTL were identified by using a collection of 63 rice varieties from Japan, and nine QTL were newly discovered [13]; 48 LTG-related QTL were detected by using 202 *O. sativa* accessions from the Rice Mini-Core (RMC) collection [4]; 42 QTLs were discovered from 421 accessions by GWA mapping, twenty-two of these QTL co-localized with a previous study [14]; 31 markers were detected for low temperature germination using 200 *japonica* rice varieties by GWAS [15]; two main QTL were identified related to LTG using a natural population comprising 137 rice cultivars and inbred lines [1]; a GWAS was conducted using 257 rice accessions from around the world and a total of 51 QTLs were identified during germination in rice [16]; and 11 QTLs for LTG were identified using 375 rice accessions selected from the Rice Diversity Panel 2 through GWAS, while 4 QTL were firstly reported [17]. A total of 53 QTL were found to be associated with LTG, of which 20 were located in previously reported QTL using 187 rice natural accessions, *OsSAP16* was identified by GWAS and it encodes a stress-associated protein containing two AN1-C2H2 zinc finger domains and acts as an essential LTG regulator [18]. To date, however, except for *OsSAP16*, few genes were found to be associated with LTG cloned by GWAS. Therefore, more germplasm resources must be used to mine more candidate genes to understand the genetic mechanism of LTG.

In our study, 211 rice accessions combine with 36,727 SNPs were used to perform a GWAS to evaluate LTG, 6 cold tolerance indices reflecting LTG were developed and assessed, a total of 18 QTL and 14 candidate genes were identified and will be useful to breeding more cold tolerance varieties.

2. Materials and Methods

2.1. Plant Material and Population Structure Analysis

All materials in this study were derived from Li. et al. [19]. A natural population of 211 rice accessions selected from International Rice Research Institute (https://www.irri.org/, accessed on 1 February 2022) was used to evaluate the six LTG-related indices (Table S1). These varieties were mainly collected from 15 different provinces in China

as well as from the Philippines and Japan. Their geographical range spans from 15° to 48° north latitude, including temperate, tropical and subtropical regions. Population structure analysis by Structure software shows that when K = 2, CV error value is the lowest, suggesting that the 211 rice accessions could be divided into two subgroups, representing *Indica* (130 accessions) and *Japonica* (81 accessions) (Figure S1A). The PCA analysis by Tassel 5.2.73 software demonstrated that the 211 rice accessions mainly formed two subgroups with different distributions along the three eigenvectors (Figure S1B). Phylogenetic analysis based on their genotypes determined by the 36,727 SNPs (https://snp-seek.irri.org/, accessed on 1 February 2022) demonstrated that the 211 rice accessions could be clustered into two subgroups (Figure S1C). Pairwise relative kinship analysis by Tassel 5.2.73 software of 211 rice landrace, the result clearly indicated that there was no strong relatedness among our population (Figure S1D). The rice materials were collected in accordance with local laws without any conflict of interest. The population was developed in the experimental field at Jiangxi Agricultural University in Nanchang, Jiangxi Province and Linwang, Hainan Province, for more than four generations.

2.2. Indices for Evaluating LTG

The seeds were placed in an oven at 45 °C for 48 h to break seed dormancy and were disinfected with sodium hypochlorite solution, then washed three times with sterile water. Each accession was represented by up to 30 seeds per Petri dish with two sheets of filter paper in a triplicate randomized block design for up to 90 seeds (3 Petri dishes) per experiment. The Petri dishes were placed in a growth incubator at 15 °C and treated in darkness for 15 days, followed by 6 days of room temperature recovery. Meanwhile, the germination experiment was carried out at room temperature as a control group. Germinated seeds were counted in each Petri dish obtained after 6 days in a room temperature growth chamber (control) and after 15 days in a 15 °C growth chamber (cold treatment), as well as the plumule lengths were recorded after 6 days in a room temperature growth chamber. Germination was defined as visible coleoptile emergence through the lemma and palea (hull). The low temperature germination percent (LTGP) was calculated as the percent of seed germination at 15 °C after 15 days. The mean LTGP index were recorded from three Petri dishes and normalized with the mean percent germinability of seeds at room temperature (NTG) which was used to calculate the relative germination percentage (RGP), RGP was determined as LTGP divided by NTG times 100. After 15 days of cold treatment, 10 germinated seeds were randomly selected from each Petri dish for coleoptile length measurement, and the average value was taken to represent coleoptile lengths after cold stress (CLC). After the recovery period of 6 days, plumule lengths of 10 germinated seedlings were randomly selected were measured and namely plumule length after 6-day recovery (PLR), the plumule length recovery rate (PLRR) index was calculated as (PLR minus CLC) divided by 6; in the control group, plumule lengths of 10 germinated seedlings were randomly selected were measured after 6 days, the relative plumule length (RPL) index was calculated as CLC divided by plumule lengths at control group, the recovery ability of plumule length after cold stress (RAPL) index was calculated as (PLR minus CLC) divided by plumule lengths at control group. All the length indexes in the experiment were manually measured by a ruler. All experiments were repeated three times.

2.3. Statistical Analysis, GWAS Mapping and Candidate Gene Analysis

The phenotypic data were sorted out by EXCEL 2010, and the correlation coefficients were calculated by SPSS 26.0 software. The GWAS analysis was performed with a line mixed model to determine the association between genotype and evaluated phenotype using Tassel 5.2.73 software. LD Block was performed to identified candidate gene regions by Haploview 4.2, the information of candidate genes was collected and classified by NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 1 February 2022), China Rice Data Center (https://www.ricedata.cn/, accessed on 1 February 2022) and Rice Genome Annotation Project (http://rice.uga.edu/index.shtml, accessed on 1 February 2022).

2.4. Gene Expression Analysis

For analysis of the expression pattern of candidate genes, total RNA was extracted from embryos collected from the seeds of cultivars (two high-LTG value accessions and two low-LTG value accessions) using an RNA extraction kit (Promega Biotech, Shanghai, http://www.promega.com.cn, accessed on 1 February 2022). First-strand cDNA synthesis was performed using HiScript II QRT SuperMix (Vazyme, http://www.vazyme.com, accessed on 1 February 2022). Gene expression levels were calculated based on the analysis of variance (ANOVA) of three technical replicates. Te *OsActin* was included as an internal control.

3. Results

3.1. Assessment of Six LTG Indices in 211 Rice Accessions

In this study, six phenotypic assays (LTGP, RGP, RPL, PLR, PLRR and RAPL) were assessed as potential QTL mapping indices reflecting LTG of rice. Large variations in LTG were observed in 211 rice accessions under 15 °C low temperature (Table S1). LTGP values ranging from 3.3 to 100.0%, with an average of 76.8%, RGP values ranging from 4.1 to 100.0%, with an average of 80.9%, the LTGP and RGP distribution in 211 rice accessions was continuous, with more in the high LTGP and high RGP side; RPL values ranging from 3.0 to 42.0%, with an average of 20.6%, PLR values ranging from 0.8 to 8.1, with an average of 4.6, PLRR values ranging from 0.1 to 1.2, with an average of 0.6 and RAPL values ranging from 0.2 to 2.5, with an average of 1.1. The values of these four indices confirm normal distribution approximately (Figure 1).

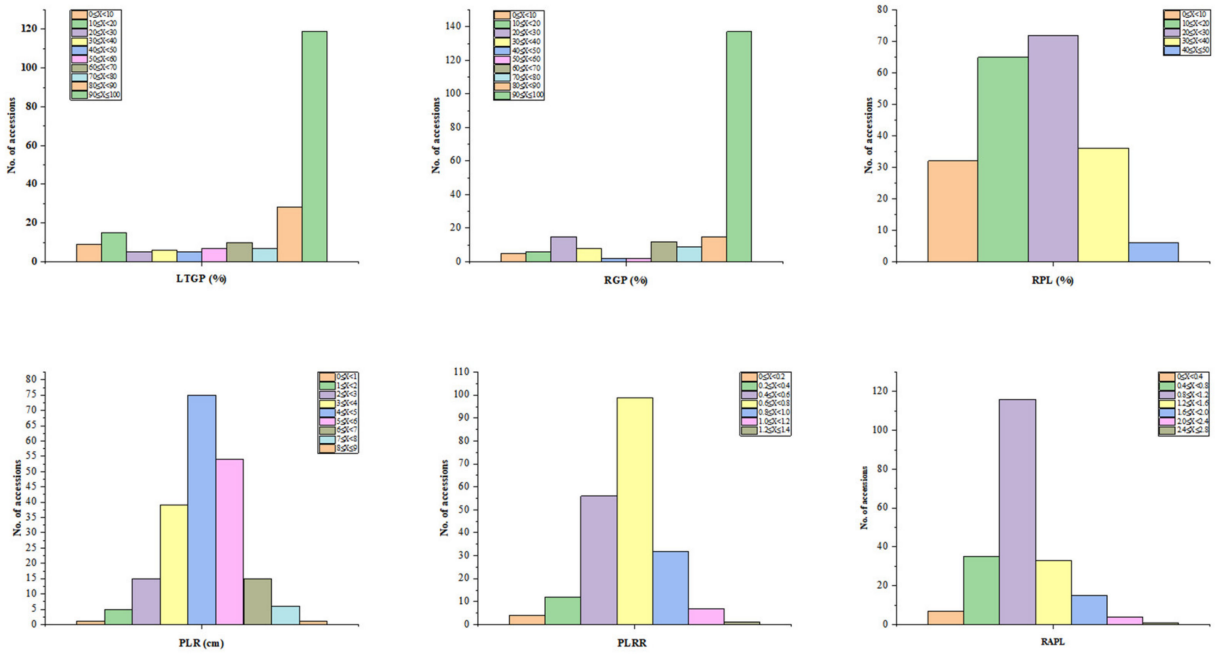

Figure 1. Distribution of six LTG indices (LTGP, RGP, RPL, PLR, PLRR and RAPL) in 211 rice accessions. The vertical axis in each subfigures represents the number of accessions, and each column on the horizontal axis represents the range of values. The LTGP and RGP distribution in 211 rice accessions was continuous while the remaining four indices are normally distributed.

The LTG indices' comparisons among different subgroups revealed that the values of LTGP, RGP, PLR and PLRR of the *indica* group was significantly higher than that of the *japonica* groups ($p < 0.01$), while the RAPL of the *japonica* group was significantly higher than that of the *indica* group; in addition, there was no significant difference in the RPL index between *indica* and *japonica* rice ($p < 0.01$) (Figure 2).

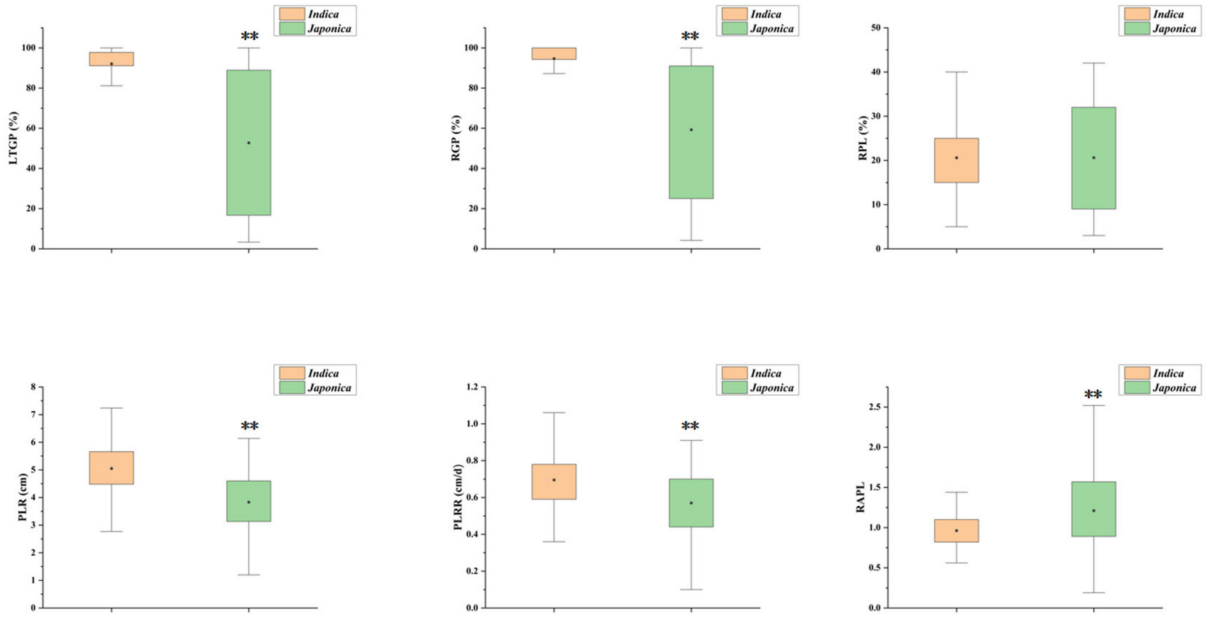

Figure 2. Comparison of LTG indices among different subgroups. The vertical axis in each subfigures represents the range of LTG index values, yellow boxes represent *indica*, green boxes represent *japonica*, and black dots in the boxes represent average values. ** Indicates significance at the 1% level.

To determine how the means of different LTG indices for each accession were compared to each other, pairwise Pearson's correlation analysis was conducted (Table 1; Figure 3). This showed LTGP was significantly correlated with the other five indices, and the correlation coefficient with RGP was the largest, which was negatively correlated with RAPL, and RGP has a similar situation to LTGP; PLR and PLRR were positively correlated significantly, and both were positively correlated with RAPL; and RPL was negatively correlated with PLRR and RAPL but did not reach a significant degree. This suggests that the RPL and RAPL indices have a relatively unique genetic program while LTGP and RGP, PLR and PLRR might share genetic pathways, respectively.

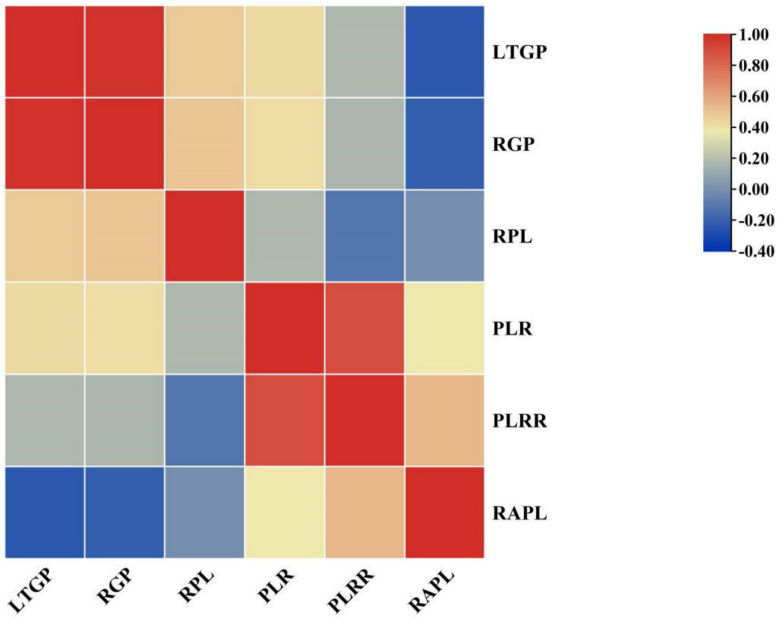

Figure 3. Heat maps of correlations between six LTG indices. Blocks that tend to be red or blue indicate greater absolute values of the correlation coefficients.

Table 1. Pearson's correlation coefficients between the six different indices evaluated in this study.

Trait	LTGP	RGP	RPL	PLR	PLRR
RGP	0.986 **				
RPL	0.483 **	0.497 **			
PLR	0.429 **	0.417 **	0.167 *		
PLRR	0.168 *	0.162 *	−0.115	0.895 **	
RAPL	−0.243 **	−0.217 **	−0.008	0.381 **	0.543 **

* Indicates significance at the 5% level; ** Indicates significance at the 1% level.

3.2. GWAS for Identification of QTLs

Based on the six indices' phenotype data and 36,727 K SNP database, PCA and KINSHIP were used as covariates for GWAS in a mixed linear model. A total of 18 QTL were identified at $p < 0.001$, with two, three, five, four, two and two QTL were discovered to be significantly associated with LTGP, RGP, PLR, PLRR, RPL and RAPL, respectively (Table 2; Figures 4 and 5). The amount of phenotypic variance explained (R^2) ranged from 6.27% to 7.08% for LTGP, 3.98% to 7.52% for RGP, 7.29% to 8.59% for RPL, 6.02% to 10.31% for PLR, 6.51% to 8.79% for PLRR and 8.79% to 11.88% for RAPL. Among these QTL, seven were discovered for the first time, *qLTGP2* and *qRGP2* were co-localized at 3.3 Mb, and *qPLR2* and *qPLRR2* were co-localized at 5.5 Mb on chromosome 2; three QTL from the three indices (*qLTGP5*, *qPLR5* and *qPLRR5*) share the same SNP peak at 27.8 Mb on chromosome 5. In addition, eleven QTL were co-localized with those from previous studies, *qPLR1* was located at 39.9 Mb on chromosome 1 overlapped with *SNAC2* [20]; *qRGP4* and *qRPL4* located at 29.7 Mb and 29.9 Mb on chromosome 4, respectively, were located at the same interval as *OsAOX1a* [21]; and *qPLR6* and *qPLRR6* were co-localized at 5.7 Mb on chromosome 6 overlapped with *OsABF2* [22]. On chromosome 9, the three QTL (*qRGP9*, *qRPL9* and *qRAPL9*) with different peak SNPs were all located in a QTL, *clr9*, with a large interval (2.6~16.3 Mb) [23], and it was further found that *qRGP9* was co-located with *OsDREB6* and *qRAPL9* was co-located with *OsWRKY76* [24,25]. *qRAPL10* overlapped with *qLTG10-1*, which harbored the highest-peak SNP, chr19057293, which explain 11.88% of the total phenotypic variation [4]. The remaining two QTL on chromosomes 12 (*qPLR12* and *qPLRR12*) were co-located with each other and overlapped with *qLTSS12-1* [4]. This further suggests LTGP-QTL and RGP-QTL, PLR-QTL and PLRR-QTL share overlapping or converging genetic mechanisms, respectively.

Table 2. Summary of the QTL identified by GWAS mapping for six LTG indices and co-localized genes and QTLs.

QTL ID	Chr.	Peak SNPs	*p* Value	R2	Previous QTLs/Genes
qPLR1	1	39993855	0.000504630	0.060240000	SNAC2 [20]
qLTGP2	2	3339289	0.000533207	0.062685322	
qRGP2		3339289	0.000111934	0.075171265	
qPLR2		5505457	0.000816111	0.069550000	
qPLRR2		5505457	0.000813330	0.068310000	
qRGP4	4	29781197	0.000007752	0.057870000	OsAOX1a [21]
qRPL4		29921285	0.000098708	0.085890000	*OsAOX1a*
qLTGP5	5	27865039	0.000206354	0.070831922	
qPLR5		27865039	0.000039949	0.096883103	
qPLRR5		27865039	0.000991230	0.074340000	
qPLR6	6	5745395	0.000021543	0.103143691	OsABF2 [22]
qPLRR6		5745395	0.000496299	0.065075903	*OsABF2*
qRGP9	9	12912697	0.000268990	0.039760000	clr9 [23]; OsDREB6 [24]
qRPL9		13641693	0.000494500	0.072930000	*clr9*
qRAPL9		15684209	0.000094869	0.087940000	clr9; OsWRKY76 [25]
qRAPL10	10	19057293	0.000001319	0.118803366	qLTG10-1 [4]
qPLR12	12	23577481	0.000142160	0.089220000	qLTSS12-1 [4]
qPLRR12		23577481	0.000120220	0.087880000	qLTSS12-1 [4]

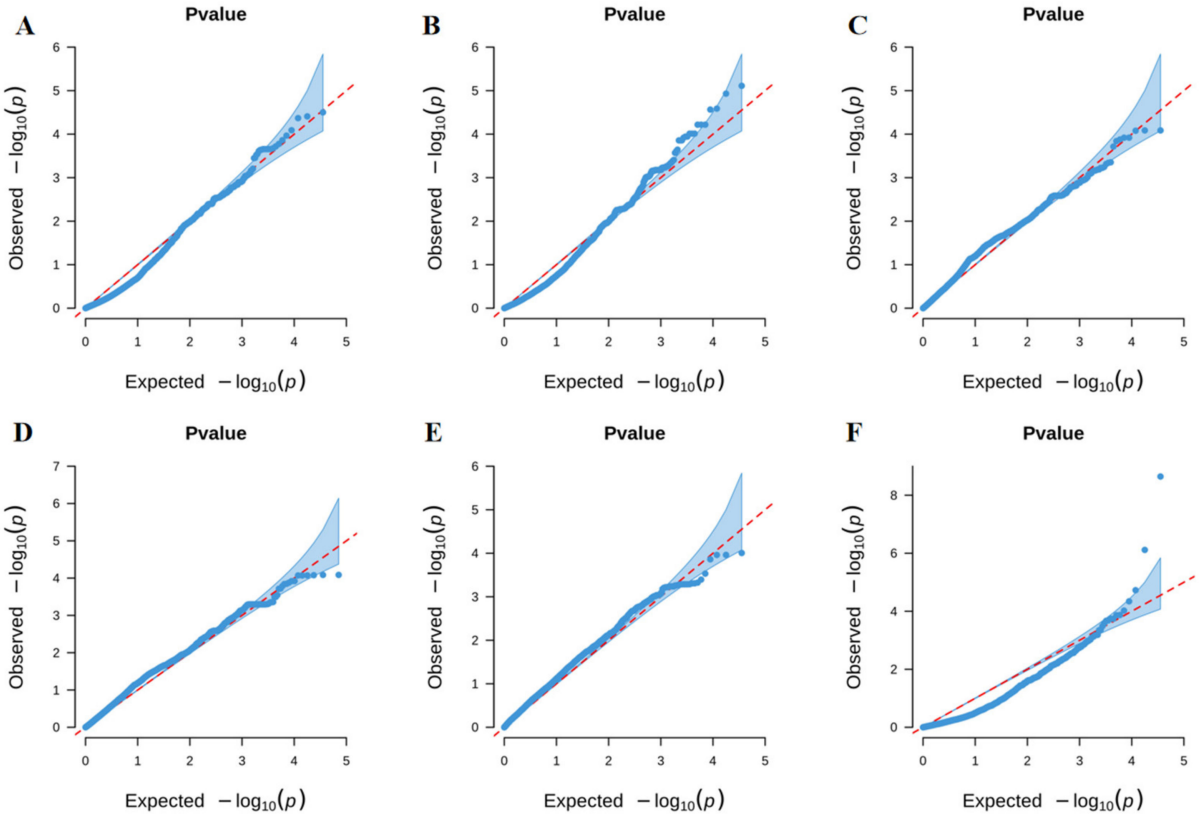

Figure 4. Quantile–quantile (Q–Q) plot of GWAS for six LTG indices (LTGP (**A**), RGP (**B**), RPL (**C**), PLR (**D**), PLRR (**E**), RAPL (**F**)).

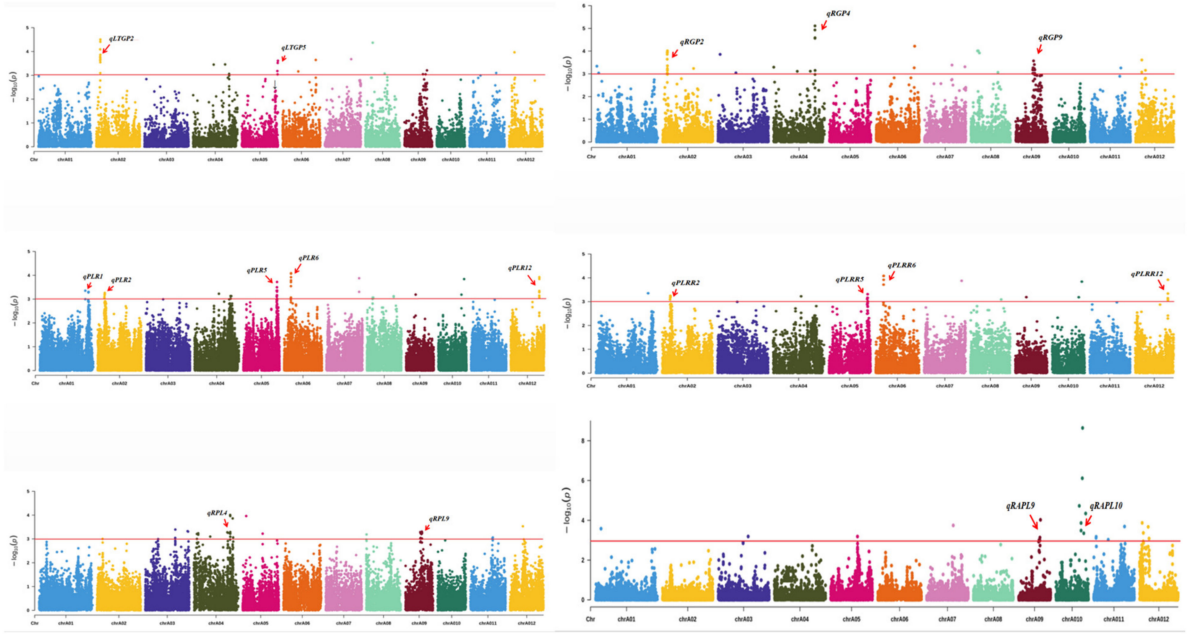

Figure 5. Manhattan plots of GWAS for six LTG indices. The vertical axis in each subfigures represents the value of -$\log_{10}(P)$, and the horizontal axis represents 12 chromosomes in rice, the solid red line represents when P value equals 0.001. The red arrow indicates QTLs detected from six indices.

3.3. Candidate Gene Analysis

As shown in Table 2, five QTL were detected in at least two indices, namely *qLTGP2* (*qRGP2*), *qPLR2* (*qPLRR2*), *qLTGP5* (*qPLR5* and *qPLRR5*), *qPLR6* (*qPLRR6*) and *qPLR12* (*qPLRR12*). Considering that a cold tolerance gene had been characterized from *qPLR6* region, *qPLR6* was excluded from candidate gene analysis. In addition, *qRAPL10* explained the largest phenotypic variation and was also considered within the scope of candidate gene analysis. According to the LD decay analysis, a total 61 kb region was identified as the candidate region for *qLTGP2*, 487 kb for *qPLR2*, 440 kb for *qLTGP5*, 353 kb for *qRAPL10* and 217 kb for *qPLR12* (Figure S2). To narrow down the candidate gene numbers, genes annotated as retrotransposons, transposons, hypothetical proteins and unknown proteins were excluded from the analysis; thus, 161 genes were obtained from the five QTL (Table S2). Furthermore, based on NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 1 February 2022), China Rice Data Center (https://www.ricedata.cn/, accessed on 1 February 2022) and Rice Genome Annotation Project (http://rice.uga.edu/index.shtml, accessed on 1 February 2022), 14 genes were screened out of 161 genes in these 5 QTL regions that may be involved in cold stress, oxidative stress or seed germination in previous studies (Table 3).

Table 3. Candidate genes of the five QTLs.

QTLs ID	Candidate Genes			
	Locus	**Gene**	**Protein**	**Description**
qLTGP2	*LOC_Os02g06592*	*CHR701*	SNF2 family N-terminal domain containing protein	Snf2 family proteins can be induced by various abiotic stresses [26].
qPLR2	*LOC_Os02g10200*	*ZFP185*	A20/AN1-type zinc finger protein	The expression level of *ZFP185* was susceptible to salt stress, osmotic stress and cold stress [27].
	LOC_Os02g10510	*OsDDI1*	Ubiquitin family domain-containing protein	*CsDDI1* is involved in the physiological and molecular mechanisms of cold acclimation [28].
	LOC_Os02g10700	*OsEBF2*	OsFBL7—F-box domain- and LRR-containing protein	*OsEBF2* involved in ethylene signaling in anthers is upregulated after cold stress in rice [29].
	LOC_Os02g10760	*OsWR1*	AP2 domain-containing protein	*OsWR1* is also a gene associated with ethylene metabolism in anthers, which is slightly downregulated after cold stress [29].
	LOC_Os02g10800	*OsBT1*	mitochondrial carrier protein	OsBT1 is involved in seed germination and regulates seed dormancy through glucose metabolism [30].
qLTGP5	*LOC_Os05g47840*	*IPT7*	tRNA isopentenyltransferase family protein	It was upregulated during the recovery stage after cold stress in cold-tolerant cultivars [31].
	LOC_Os05g47890	*OsRACK1B*	WD domain, G-beta repeat domain-containing protein	It may be involved in rice seed germination by regulating G protein to control hormone signaling response [32].
	LOC_Os05g48020	*OsSYP71*	SNARE domain-containing protein	It was significantly upregulated under oxidative stress [33].
	LOC_Os05g48390	*OsPHO2*	Ubiquitin-conjugating enzyme protein	After cold acclimation, arabidopsis *pho*2 mutants, with increased stem Pi, were more sensitive to freezing than the wild type [34].
	LOC_Os05g48590	*IAA19*	OsIAA19—Auxin-responsive Aux/IAA gene family member	IAA19 is an auxin-related gene that is induced by cold stress [31].
qRAPL10	*LOC_Os10g35810*		thylakoid lumenal protein	It induced during the chilling and recovery treatment periods of 9311 and DC90 [35].
qPLR12	*LOC_Os12g38200*	*OsDof29*	dof zinc finger domain-containing protein	It is upregulated after cold stress [36].
	LOC_Os12g38400	*OsMYB91*	MYB family transcription factor	It is a stress responsive gene that participates in the coordination of abiotic stress resistance [37].

3.4. Gene Expression Analyses of Candidate Genes

Fourteen candidate genes were selected to compare expression levels between high-LTG value (BK022 and BK028) and low-LTG value accessions (BK202 and BK205) by qRT-PCR analysis. In these analyses, four genes (*LOC_Os02g06592*, *LOC_Os02g10700*, *LOC_Os02g10760* and *LOC_Os05g48590*) were differentially expressed between cold treatment and normal growth conditions (Figure 6). These genes showed higher transcript

levels in cold treatment than in normal growth conditions. Particularly, the expression level of *LOC_Os05g48590* in two varieties with low-LTG values increased more significantly than that in two varieties with high-LTG values after cold treatment. Further experiments including genetic complementation analyses should be conducted to verify the gene controlling cold tolerance at the germination stage.

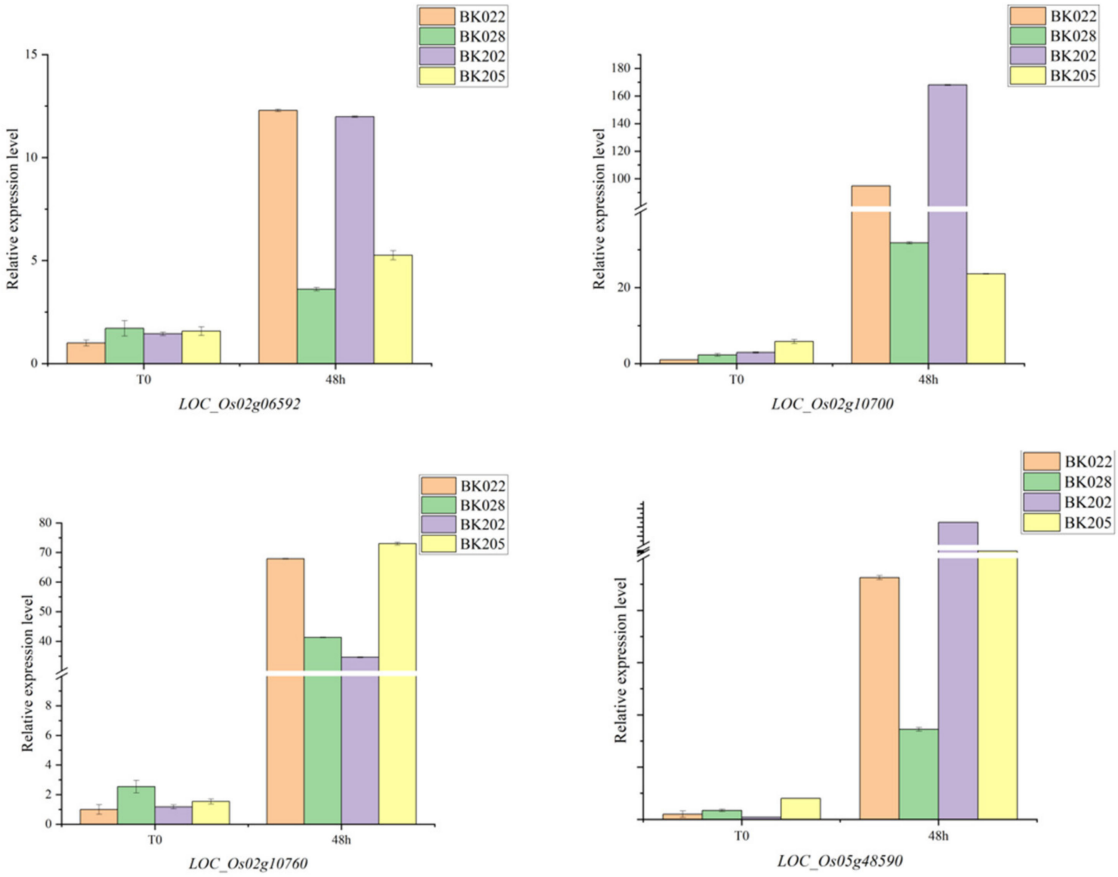

Figure 6. Expression patterns of four candidate genes. The vertical axis in each subfigures represents the relative expression level of gene, and the horizontal axis represents different treatments i.e., T0 is the control treatment and 48h is the 48h after cold treatment. Different colored columns represent different accessions of rice.

4. Discussion

In our study, six indices from two stages were used to evaluate LTG, namely LTGP, RGP and RPL in the cold treatment stage and PLR, PLRR and RAPL in the recovery stage. Overall, *indica* rice had a higher germination rate than *japonica* rice at room temperature [38], so separate GWAS analyses of LTGP and RGP helped us to discover whether cold tolerance was due to inherent cold tolerance or high seedling vigor. Interestingly, in this study, we found that LTGP and RGP values of *indica* rice were higher than those of *japonica* rice, it appears that the germination percent of *indica* rice under cold stress is higher than that of *japonica* rice, which was consistent with the results of Yang et al. [16], but also opposite to the results of Cui et al. [39], Morsy et al. [40] and Lv et al. [41]. Moreover, some studies showed that there was no significant difference in LTG between *indica* and *japonica*, and it was speculated that *indica* might gradually adapt to low temperatures during the germination process [4,15], the RPL index in this study showed that there were no significant differences between *indica* and *japonica*.

In the recovery stage, the PLR and PLRR values of *indica* rice were both larger than those of *japonica* rice, but the RAPL values were opposite, indicating that *japonica* rice

showed a stronger recovery ability than *indica* rice in the recovery stage (Figure 7), this is similar to the results of previous studies [4], that is, higher LTGP values do not represent stronger recovery ability, and the cold treatment stage and recovery stage may be controlled by different genetic mechanisms. LTGP was observed to be strongly correlated with RGP, and the same situation same was observed between PLR and PLRR (Table 1; Figure 3), suggesting that LTGP and RGP might share genetic pathways, and PLR and PLRR are in the same way. On the other hand, RAPL was negatively correlated with LTGP and RGP, and positively correlated with PLR and PLRR, but the correlation coefficients were not large, suggesting that RAPL may be controlled by an independent genetic mechanism, this was confirmed in our GWAS analysis.

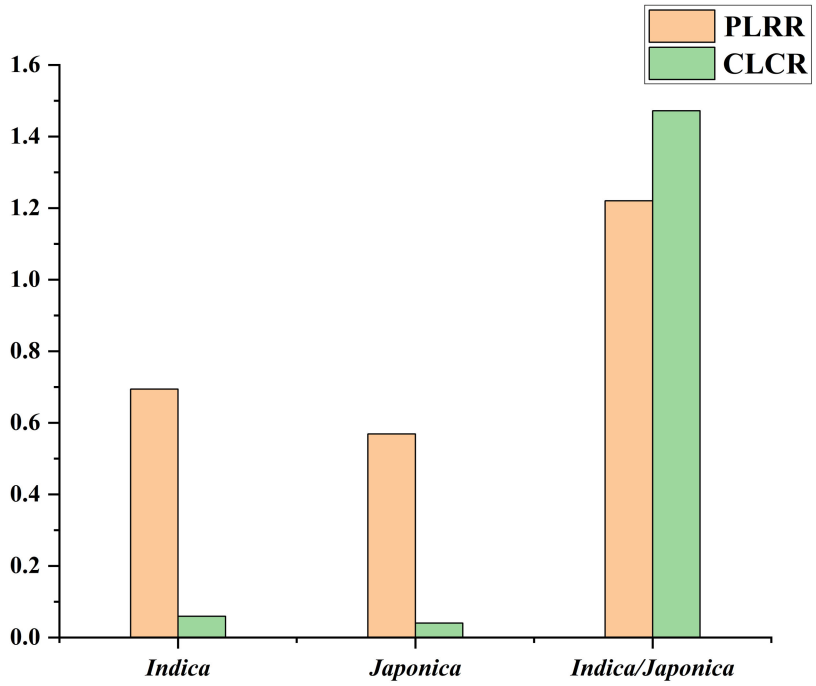

Figure 7. Comparison of PLRR and CLCR (CLC divided by 15) indices among different subgroups. Yellow boxes represent *indica*, green boxes represent *japonica*.

In our study, several identified QTLs were found to overlap with QTLs/genes previously studied. The candidate region for *qPLR1* was found to contain a cloned cold-tolerant gene, *SNAC2*. *SNAC2* encodes a plant-specific NAC transcription factor that is induced by drought, salinity, cold, mechanical damage and ABA treatment. Transgenic rice with the overexpression of *SNAC2* had higher tolerance to cold stress, salt stress and PEG treatment [20]. The two QTLs, *qRGP4* and *qRPL4*, were co-located with a cloned cold-tolerant gene, *OsAOX1a*. In SDS gel hybridization of rice callus protein, the varieties without low temperature tolerance QTL showed 32 kDa AOX band, while the varieties with QTL showed 34 kDa AOX band. This variation is attributed to single nucleotide polymorphisms between *OsAOX1a* alleles, causing Lys^{71} to replace Asn^{71} [21]. On chromosome 6, two QTLs (*qPLR6* and *qPLRR6*) were found to share the same peak SNP, and a cloned gene *OsABF2* was found in their candidate regions. *OsABF2* is expressed in many tissues of rice and is induced by several abiotic stresses such as drought, saline-alkali, low temperature, oxygen stress and ABA stress [22]. On chromosome 9, three identified QTLs (*qRGP9*, *qRPL* and *qRAPL9*) with different peak SNPs were found to be contained in *clr9*, which is a QTL associated with the culm length growth rate under cold stress [23]. A QTL on chromosome 10 and a co-locus on chromosome 12 were found to overlap with the results of a previous study on LTG [4], that is, *qRAPL10* overlapped with *qLTG10-1* and *qPLR12* (*qPLRR12*) overlapped with *qLTSS12-1*. In a conclusion, there is a lot of overlap between our QTL mapping results and previous studies, which reveals the reliability of our research

results. In addition, several new QTLs were excavated in this study, and it is necessary for these new QTLs to be further screened for candidate genes to obtain more casual cold tolerance genes.

GWAS analysis and LD Block analysis showed that five QTL were simultaneously detected in at least two indices (Table 2; Figure S2). The QTL, *qLTGP2* for LTGP, was observed co-localized with *qRGP2* for RGP. This is a new QTL, and according to LD Block analysis, nine genes were found in this QTL (unknown proteins have been excluded), and *LOC_Os02g06592* was considered as a possible candidate gene, which belongs to the SWI2/SNF2 family and could be induced by various abiotic stresses [26]. *LOC_Os02g10200* (*ZFP185*) [27], *LOC_Os02g10510* (*OsDDI1*) [28], *LOC_Os02g10700* (*OsEBF2*) [29], *LOC_Os02g10760* (*OsWR1*) [29] and *LOC_Os02g10800* (*OsBT1*) [30] were considered as possible candidate genes for *qPLR2* (*qPLRR2*). *LOC_Os02g10200* is constitutively expressed in multiple tissues, including roots, stems, leaves and panicles. Under salt stress, the expression of *LOC_Os02g10200* increased slightly first and then decreased. After osmotic treatment, the expression of *LOC_Os02g10200* was firstly increased and then decreased. Cold stress induced the expression of *LOC_Os02g10200*, while the expression of *LOC_Os02g10200* changed little after ABA treatment. *LOC_Os02g10510* is a homologous gene of *CsDDI1* in rice, and *CsDDI1* is involved in the physiological and molecular mechanisms of cold acclimation-induced postharvest cold tolerance in cucumber. The gene *LOC_Os02g10700* involved in ethylene signaling in anthers is upregulated after cold stress in rice, and it is also a gene associated with ethylene metabolism in anthers, which is slightly downregulated after cold stress. *LOC_Os02g10800* is involved in seed germination and regulates seed dormancy through glucose metabolism, independent of GA and ABA pathways. *LOC_Os05g47840* (*IPT7*) [31], *LOC_Os05g47890* (*OsRACK1B*) [32], *LOC_Os05g48020* (*OsSYP71*) [33], *LOC_Os05g48390* (*OsPHO2*) [34] and *LOC_Os05g48590* (*IAA19*) [31] were considered as possible candidate genes for *qLTGP5* (*qPLR5* and *qPLRR5*). *LOC_Os05g47840*, the gene encoding rate-limiting enzyme of cytokine biosynthesis, was upregulated in the roots of TNG67 (cold tolerant), but not in the roots of TCN1 (cold sensitive) during the recovery stage; *LOC_Os05g48590* (*IAA19*) is an auxin-related gene that is induced by cold stress in both TNG67 and TCN1. *LOC_Os05g47890* may be involved in rice seed germination by regulating G protein to control hormone signaling response. The expression of *LOC_Os05g48020* was significantly upregulated under oxidative stress or inoculation, and overexpression of *LOC_Os05g48020* enhanced the tolerance of rice to oxidative stress and rice blast. The expression of *LOC_Os05g48390* was downregulated with the downregulation of *OsSPX1*. The mutant of *LOC_Os05g48390* showed major leaf tip necrosis and increased Pi uptake and transport. After cold acclimation, *arabidopsis pho2* mutants, with increased stem Pi, were more sensitive to freezing than the wild type. *LOC_Os10g35810* was considered as a possible candidate gene for *qRAPL10* and induced during the chilling and recovery treatment periods of 9311 and DC90. *LOC_Os12g38200* (*OsDof29*) and *LOC_Os12g38400* (*OsMYB91*) were considered as possible candidate genes for *qPLR12* (*qPLRR12*). *LOC_Os12g38200* belongs to the C2C2-Dof family and was upregulated in the DN under control (CKDN) vs. DN under low-T w (D15DN) group. *LOC_Os12g38400* participates in the coordination of abiotic stress resistance by regulating the expression of *SLR1*. Overexpression of *LOC_Os12g38400* decreased plant growth and seed germination and growth sensitivity to ABA. Otherwise, qRT-PCR was performed to evaluated expression level of 14 candidate genes. The result showed that four genes were differentially expressed between cold treatment and normal growth conditions, the mining of these genes will help to cultivate low-temperature-tolerant rice.

5. Conclusions

In the present study, we performed GWAS analysis and identified QTL for LTG in the natural population. Among the 18 QTL for LTG, five QTL were detected in at least two indices. From these five QTL, 14 genes were candidates, and four of them were differentially expressed between cold treatment and normal growth conditions. The highlight of this

study is the combination of previously unstudied rice accessions with the GWAS strategy and the discovery of a number of new QTL related to LTG in rice that will assist in the breeding of new rice varieties with cold tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12051194/s1, Table S1. Phenotypic data and information of the 211 rice accessions; Table S2. Genes annotation from the five QTLs; Figure S1. Population structure analysis 211 rice accessions. (A) K values plotted as the number of subgroups; (B) Principal component analysis of 211 rice accessions, each black dots represents an accession; (C) Neighbor-joining tree based on Nei's genetic distances; (D) Pairwise relative kinship analysis of 211 rice accessions; Figure S2. LD Block for the five QTLs. (A) LD Block for qLTGP2; (B) LD Block for qLTGP2; (C) LD Block for qLTGP5; (D) LD Block for qRAPL10; (E) LD Block for qRLR12.

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