Identification of QTLs involved in cold tolerance during the germination and bud stages of rice (*Oryza sativa* L.) via a high-density genetic map

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Low-temperature tolerance during the germination and bud stages is an important characteristic of direct-seeded rice (DSR). Recombinant inbred lines (RILs) derived from *indica* rice H335, which is highly tolerant to low temperature, and *indica* rice CHA-1, which is sensitive to low temperature, were used to identify quantitative trait loci (QTLs) associated with low-temperature tolerance during the germination and bud stages. A total of 11 QTLs were detected based on a high-density genetic map; among these, six QTLs explained 5.13–9.42% of the total phenotypic variation explained (PVE) during the germination stage, and five QTLs explained 4.17–6.42% of the total PVE during the bud stage. All QTLs were distributed on chromosome 9, and all favourable alleles originated from H335. The physical position of each QTL was determined, and 11 QTLs were combined into five genetic loci; three of these loci are involved during the germination stage (loci 1, 2, and 3), and three are involved during the bud stage (loci 3, 4, and 5). Loci 2, 4 and 5 were repeatedly detected in the wet season (WS) and dry season (DS). Notably, loci 3 was detected during both the germination and bud stages. These loci are good candidates for future studies of gene function and could serve as highly valuable genetic factors for improving cold tolerance during the germination and bud stages of rice.

**Key Words:** rice, germination, bud, cold tolerance, QTL mapping.

Introduction

Rice (*Oryza sativa* L.) is the most important cereal crop species and the principal food of nearly 50% of the global population (Wei et al. 2013). Cultivars with high yield potential and robust and durable resistance to both biotic and abiotic stresses need to be developed to meet the increasing demand for rice (Khush 1999). Low temperature or cold is a worldwide problem that limits rice yields. Low temperatures during germination and seedling growth can lead to reduced germination, increased seedling mortality, seedling stunting, leaf yellowing or wilting, which subsequently results in lower yields (Andaya and Tai 2006, Fujino 2004, Lou et al. 2007). For example, cold damage occurs in all rice-growing areas in China, and major cold damage occurs every 4 to 5 years and results in an annual loss of 5–10 billion kg of rice in each of these disaster years (Cai and Zhang 2018). Recently, the use of direct-seeded rice (DSR) has become increasingly popular worldwide due to its cost efficiency and convenience (Mahender et al. 2015). However, rice seeds that are directly planted into soil or pre-germinated seeds that are sown in fields flooded with cool irrigation water (water-seeded) are more susceptible to cold stress. Therefore, low-temperature sensitivity at the germination and bud stages remains a major challenge for DSR. Improved cold tolerance at the germination and bud stages is an important agronomic characteristic in DSR breeding programmes.

Due to the complexity and polygenic nature of cold tolerance, considerable efforts have been directed towards the detection and mapping of quantitative trait loci (QTLs). Various low-temperature germinability-related QTLs have been identified in different populations (Chen et al. 2006, Cui et al. 2002, Fujino 2004, Hou et al. 2004, Ji et al. 2008, Jiang et al. 2006, 2017, Li et al. 2013, Miura et al. 2001, Satoh et al. 2016, Teng et al. 2001, Wang et al. 2011, 2018). For example, Miura et al. (2001) identified five low-temperature germinability-related QTLs on chromosomes 2, 4, 5, and 11. Jiang et al. (2006) found 11 putative QTLs associated with low-temperature germinability on chromosomes 3, 4, 5, 7, 9, 10, and 11. Ji et al. (2008) performed a low-temperature germinability genetic analysis and identified a total of 11 QTLs on chromosomes 2, 5, 7, 8, 11, and 12. Wang et al. (2011) identified two minor QTLs responsible for cold tolerance during germination, and Li et al. (2013) discovered three QTLs associated with low-temperature germination. Borjas et al. (2016) identified 49...
QTLs for 11 traits distributed across 10 chromosomes. Satoh et al. (2016) detected four QTLs responsible for low-temperature germinability, and Jiang et al. (2017) detected six QTLs in a back-cross inbred line (BIL) population derived from a japonica × indica cross.

Fewer QTLs have been identified at the bud stage than at the germination stage. By analysing previously reported results, Yang et al. (2015) reported 29 QTLs related to cold tolerance at the bud stage. For example, Chen and Li (2005) constructed a recombinant inbred line (RIL) population derived from an indica-japonica cross and mapped four cold tolerance-related QTLs at the bud stage on chromosomes 1, 3, 7 and 11. Qiao et al. (2005) mapped three QTLs on chromosomes 2, 4 and 7, and Zhang et al. (2007) detected three QTLs on chromosomes 5 and 12. Yang et al. (2009) detected four QTLs on chromosomes 3, 7 and 12. Using 95 chromosome segment substitution lines constructed from indica rice variety 9311 and japonica rice variety Nipponbare, Lin et al. (2010) studied cold tolerance at the bud stage and detected three QTLs and one QTL on chromosomes 5 and 7, respectively. However, among the QTLs identified at the germination and bud stages, only two QTLs have been cloned. The first QTL was qLTG3-1, which contains a gene that encodes a protein with unknown function (Fujino et al. 2008). The second QTL was OsSAP16, which contains a gene that encodes a zinc-finger domain protein and serves as the major causal gene for low-temperature germinability (Wang et al. 2018).

After summarizing the above results, we found that few genes have been finely mapped and cloned because these low-temperature germinability-related QTLs were identified based on traditional markers, such as simple sequence repeats (SSRs) and restricted fragment length polymorphisms (RFLPs), and were consistently sparsely distributed across the 12 rice chromosomes, which reduces the efficiency of mapping research. Due to the development of sequencing technology, high-density single-nucleotide polymorphisms (SNPs) are becoming increasingly available, and the genomic information provided is expected to bridge the gap between QTLs and candidate genes (Kooke et al. 2016, Mao et al. 2015, Si et al. 2016). In this study, a high-density genetic map consisting of 2498 bin markers obtained via the sequencing-based genotyping of 275 RILs derived from two indica parents was used for QTL mapping.

The breeding of cold-tolerant varieties might be the most effective way to prevent cold damage in rice. The marker-assisted back-crossing strategy is the most common and effective strategy for variety improvement. This strategy involves the transferring of a specific allele at a target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome (Li et al. 2013). The differentiation of the two major domesticated rice subspecies (indica and japonica) is associated with temperature: japonica is tolerant to low temperature, whereas indica is sensitive to low temperature (Zhao et al. 2013). Hence, japonica accessions are normally used as donors to provide cold tolerance when breeding for improved cold tolerance (Mackill and Lei 1997). However, due to the large differences in material backgrounds, the number of back-cross generations needs to be increased. Therefore, it is necessary to identify indica rice materials that show high tolerance to cold for use as donors to improve the cold tolerance of indica rice, which could effectively solve this problem. Here, we investigated the cold-tolerant germinability of two indica rice lines, CHA-1 and H335, and approximately 50 japonica rice lines from northern China and Japan. The results showed that the cold-tolerant germinability of H335 was equal to that of our japonica rice varieties, whereas CHA-1 exhibited poor cold-tolerant germinability. Therefore, we created a high-generation RIL population with CHA-1 and H335 as the parents and identified cold tolerance-related QTLs at the germination and bud stages via a high-density genetic map constructed through a genome sequencing approach. We aimed to identify cold tolerance-related QTLs from indica rice to further understand the genetic basis of cold tolerance at the germination and bud stages and to provide favourable materials and genetic resources for DSR breeding programmes.

### Materials and Methods

#### Plant materials

We constructed an RIL population consisting of 275 lines derived from an H335 and CHA-1 cross using the single-seed descent method. The RILs were grown in a paddy field at South China Agricultural University, Guangzhou, China (at approximately 113° E longitude and approximately 23° N latitude), during the wet season (WS; F6) and dry season (DS; F7) in 2017. Each RIL or parent was planted in a block design (6 columns × 6 rows) with a spacing of 20 cm between plants. Crop management and disease and insect pest control were performed in accordance with local recommendations. All materials were obtained from the germplasm resource bank of the National Engineering Research Center of Plant Space Breeding. Considering the effects of seed maturity, six individual plants in the middle of each block were harvested independently on the 35th day after heading during the WS and on the 40th day after heading during the DS. The harvested seeds were dried in a heated air dryer at 42°C for 5 days and then stored at −20°C. The cultivation and treatment methods used for the 50 japonica rice lines were the same as those used for the RIL population during the DS.

#### Phenotypic evaluation

Seeds from three plants were selected, placed in an oven at 50°C, and treated with dry heat for 7 days to break dormancy. All the seeds were surface sterilized with 20% bleach (6–7% NaOCl) for 20 min and then rinsed three times with sterile distilled water. Germination was defined
as the emergence of the coleoptile from the seed (Fujino 2004, Fujino et al. 2008). Thirty seeds per plant were placed on filter paper in a 9-cm Petri dish, and 10 ml of distilled water was then added. All Petri dishes were subsequently placed in a growth chamber with a temperature of 15°C and a 12-h light (200 μmol m⁻² s⁻¹)/12-h dark photoperiod. The number of germinated seeds was determined every 2 days. After 8 days, all the Petri dishes were transferred to a chamber at 30°C for 2 days, and the number of seeds that eventually germinated was determined. The germination rates of the seeds after 2, 4, 6 and 8 days under low-temperature conditions were calculated as follows: germination rate (%) = (number of germinated seeds during the test period/total number of germinated seeds) × 100. The low-temperature germination index was calculated as Σ(Gt/Dt), where Gt is the germination percentage on each day and Dt is the number of LTG days (Ji et al. 2008).

To evaluate the cold tolerance at the bud stage, we followed the abovementioned method used for assessment of the cold tolerance prior to germination. Three plants of each RIL were selected, i.e., three biological replicates per RIL. Thirty buds (whose length was approximately 0.5–1 cm) per individual were selected and placed in a 9-cm Petri dish, and 10 ml of distilled water was then added. All the Petri dishes were placed in a dark incubator with a temperature of 4°C for 4 days and then subjected to 30°C under a 12-h light (200 μmol m⁻² s⁻¹)/12-h dark photoperiod to restore growth. After the seedlings were allowed to recover for 7 days, we counted the numbers of normal seedlings, aberrant seedlings and dead seedlings. The standards used for the evaluation of bud cold tolerance were calculated using the following three formulas: normal seedling rate (%) = (number of normal seedlings/30) × 100; aberration rate (%) = (number of aberrant seedlings/30) × 100; and survival rate (%) = [(30 – number of dead seedlings)/30].

QTL analyses

Young leaves (F6) were collected from the seedlings of 275 RILs, and their genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Healey et al. 2014). SNP calling and bin map construction were performed using previously reported methods (Huang et al. 2009, Xie et al. 2010). The genotypes were called based on the SNP ratios. The breakpoints were determined at the boundary of the different genotypes, and bin markers were obtained by combining genotypes with recombination breakpoints (unpublished). The resequencing data of the RILs and subsequent linkage map data are not shown. QTL IciMapping v4.1 software (Meng et al. 2015) was used for the QTL analyses. The inclusive composite interval mapping (ICIM-ADD) method was used for the identification of QTLs via the BIP function using the following parameters: the window size was 1 cM, and the threshold for the logarithm of odds (LOD) scores was set to 2.5. The identification of epistatic QTLs was performed using the inclusive composite interval mapping of epistatic (ICIM-EPI) method, and the threshold for the LOD scores was set to 5.0. The proportions of phenotypic variation explained (PVE) accounted for by each QTL and each pair of epistatic QTLs as well as the corresponding additive effects were also estimated. The QTL nomenclature followed that described by McCouch and CGSNL (2008).

Results

Phenotypic analysis of the parental lines and RILs

The germination index can be used to evaluate the speed of germination comprehensively and effectively. Hence, we investigated 50 japonica rice cultivars using the low-temperature germination index. Their average low-temperature germination index was 31.63 during the WS and 37.39 during the DS (Supplemental Table 1). The low-temperature germination index of our low-temperature tolerant parent material (H335) was 25.38 during the WS and 26.93 during the DS, and the low-temperature germination index of the low-temperature germination-sensitive parent material (CHA-1) was 14.48 during the WS and 17.53 during the DS. These findings indicate that similarly to japonica rice, H335 exhibits favourable low-temperature tolerance and might carry favourable genes associated with tolerance to low-temperature germination. Notably, all eight trait indicators were investigated in the samples obtained during the two seasons, and the two parents exhibited highly significant differences in these traits (Table 1, Supplemental Table 2). Within the population, all eight traits varied significantly, and RILs with trait values that were higher and lower than the trait values of their high-value and low-value parents, respectively, emerged, which suggested that transgressive segregation might have occurred in the population. In addition, although these traits did not present a typical normal distribution, we still determined that these traits were quantitative genetic traits (Table 1, Fig. 1).

We also assessed all pairwise correlations among these eight traits (Table 2). The WS and DS results were approximately the same, and no strong correlations were found between traits related to low-temperature tolerance at the seed germination and bud stages. During the WS, the highest correlation coefficient (0.194) was found between the germination index and the normal seedling rate, whereas the highest correlation coefficient during the DS (only 0.126) was found between the germination rate on the second day and the normal seedling rate. As expected, strong correlations were found between the five traits at the germination stage and between the three traits at the bud stage. During the germination stage, the greatest correlation during both the WS and the DS was obtained between the germination rate and the germination index on the fourth day, with values of 0.897 and 0.862, respectively. At the bud stage, the highest correlation coefficient during the WS (0.760) was found between the survival rate and the normal seedling rate, whereas the highest correlation coefficient
QTL mapping within the RIL population

In a previous study (unpublished), we validated 100,307 biallelic homozygous SNPs for the determination of recombinant events using a genotyping-by-sequencing approach. A high-density bin map with an average physical length of 149.38 kb was obtained using the sliding window approach. This map contained 2498 bin markers that were evenly distributed across the 12 chromosomes. The number of markers on each chromosome ranged from 138 to 316, the total genetic distance of the linkage map was 2371.84 cM, and the average interval between markers was 0.95 cM (Figs. 2, 3). The lowest total marker density (0.62 cM marker⁻¹) was found in the linkage group on chromosome 2, whereas the greatest marker density (1.77 cM marker⁻¹) was found in the linkage group on chromosome 10.

A total of six QTLs were detected using the ICIM-ADD method with IciMapping v4.1 software in conjunction with a high-density map across the two environments: three QTLs were detected for the WS, and three were detected for the DS. Notably, for the 10 traits in the WS and DS, QTLs associated with the germination rate on the 6th and 8th days, respectively, were not detected, but QTLs associated with the germination rate and germination index on the 2nd and 4th days, respectively, were detected. Among the six QTLs, the highest PVE (9.42%) was found for *qLTGR4d-9-1*, and the lowest PVE was obtained for *qLTGR4d-9-2* (5.13%). All the QTLs were distributed on chromosome 9, and all additive effects of QTL were positive, which indicated that all favourable QTL alleles that provide cold germinability originated from H335. The QTLs that overlapped based on their physical position were classified as the same loci. In total, three loci were ultimately obtained. Notably, loci 2 (*qLTGR4d-9-2, qLTGR2d-9-1*, and *qLTGI-9-1*) was associated with different traits (the low-temperature germination rate on days 2 and 4 and the low-temperature germination index) and was repeatedly detected during the WS and DS, and loci 3 (*qLTGR2d-9-2* and *qLTGI-9-2*) was associated with different traits (the low-temperature germination rate on day 2 and the low-temperature germination index) during the WS (Table 3, Fig. 3). By comparing these loci with reported genetic loci, we found that loci 2 overlapped with *qLTG-9* (Ji et al. 2008). These findings also further indicate that our mapping results are accurate and reliable.

Similarly, six traits associated with cold tolerance at the bud stage during the WS and DS were mapped and analysed, and a total of five QTLs were identified. During the WS, one QTL associated with the survival rate and another QTL associated with the normal seedling rate under cold stress were detected. During the DS, two QTLs associated with the survival rate were detected, and one QTL associated with the normal seedling rate under cold stress was detected. However, no QTLs associated with the aberration rate during the WS or DS were detected. Among the five QTLs, the highest PVE (6.42%) was obtained for *qLTSR-9-1*, and the lowest PVE (4.17%) was found for *qLTSR-9-2*. Interestingly, similar to the findings obtained at the germination stage, all QTLs associated with the bud stage were distributed on chromosome 9, and their favourable QTL alleles that provide cold germinability originated from H335. The physical position of each QTL was determined, and a total of three loci were detected. Loci 4 (*qLTSR-9-1*) and loci 5 (*qLTNSR-9*) were repeatedly detected during the DS (0.743) was obtained between the survival rate and the aberration rate.

### Table 1. Performance of parental and RIL populations at the germination and bud stages under cold stress

| Trait                  | Env<sup>a</sup> | Parents<sup>b</sup> | RIL population | \(\text{Range} \) | \(\text{Skewness} \) | \(\text{Kurtosis} \) | \(\text{CV}^c \) (%) |
|------------------------|-----------------|----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Germination rate-2 d (%) | WS              | 2.26 ± 1.96          | 8.93 ± 1.69*    | 2.50            | 0.00–33.50      | 3.01            | 10.15           | 205.56          |
| Germination rate-2 d (%) | DS              | 2.62 ± 2.27          | 11.60 ± 1.06**  | 5.76            | 0.00–55.71      | 2.53            | 8.74            | 132.98          |
| Germination rate-4 d (%) | WS              | 16.90 ± 3.68         | 85.12 ± 0.87**  | 44.18           | 0.00–94.78      | –0.10           | 0.70            | 52.08           |
| Germination rate-4 d (%) | DS              | 22.58 ± 5.68         | 88.40 ± 1.06**  | 64.15           | 7.47–96.78      | –0.74           | –0.40           | 28.85           |
| Germination rate-6 d (%) | WS              | 67.43 ± 1.33         | 92.09 ± 3.48**  | 87.72           | 20.00–100.00    | –2.47           | 8.10            | 15.22           |
| Germination rate-6 d (%) | DS              | 86.60 ± 2.84         | 100.00 ± 0.00** | 94.49           | 45.38–100.00    | –5.89           | 52.19           | 7.07            |
| Germination rate-8 d (%) | WS              | 77.55 ± 1.53         | 97.74 ± 0.28**  | 98.09           | 57.25–100.00    | –9.76           | 118.61          | 4.47            |
| Germination rate-8 d (%) | DS              | 91.07 ± 1.89         | 100.00 ± 0.00** | 99.21           | 84.89–100.00    | –14.72          | 232.28          | 1.79            |
| Germination index       | WS              | 14.48 ± 0.96         | 25.38 ± 0.37**  | 20.22           | 8.32–32.94      | –0.45           | 3.98            | 16.76           |
| Germination index       | DS              | 17.53 ± 1.36         | 26.93 ± 0.18**  | 23.12           | 14.14–38.58     | –0.42           | 7.60            | 14.05           |
| Normal seedling rate (%)| WS              | 10.68 ± 3.09         | 31.80 ± 7.12**  | 17.77           | 0.00–100.00     | 1.68            | 1.78            | 146.24          |
| Normal seedling rate (%)| DS              | 10.89 ± 3.98         | 33.57 ± 3.50**  | 16.43           | 0.00–100.00     | 1.80            | 2.01            | 164.55          |
| Aberration rate (%)     | WS              | 3.74 ± 1.46          | 34.35 ± 2.54**  | 22.55           | 0.00–87.66      | 0.84            | –0.10           | 98.50           |
| Aberration rate (%)     | DS              | 5.34 ± 1.44          | 33.59 ± 2.05**  | 21.42           | 0.00–95.45      | 1.18            | –0.01           | 136.36          |
| Survival rate (%)       | WS              | 14.42 ± 4.53         | 68.37 ± 7.65**  | 40.33           | 0.00–100.00     | 0.33            | –1.23           | 84.69           |
| Survival rate (%)       | DS              | 16.23 ± 2.69         | 64.93 ± 1.86**  | 37.85           | 0.00–100.00     | 0.44            | –1.56           | 105.81          |

<sup>a</sup> Environment: WS represents the wet season in 2017, and DS represents the dry season in 2017.

<sup>b</sup> Parent refers to the means ± standard deviations (SDs) of the parents, and ** indicates significance at the 0.01 level.

<sup>c</sup> CV: coefficient of variation.
detected during the WS and DS. Notably, we found that loci 3 was detected at both the germination and bud stages (Table 4, Fig. 3).

Analysis of the effects of the five loci

To clarify the effects of these five loci, we calculated the phenotypic differences between the two alleles at each locus within the RIL population (Table 5). We first divided the RILs of the population into the H335 type and the CHA-1 type at each locus and then compared the differences between the two genotypes for the corresponding traits. The results showed that the mean phenotypic values of the corresponding traits of the RILs harbouring favourable alleles were significantly greater than those of the RILs harbouring nonexcellent alleles ($p < 0.01$). Although some traits were associated with a QTL during only one of the seasons, extremely significant differences between H335- and CHA-1-type RILs across both seasons were detected.

To verify the reliability and breeding value of the detected loci further, we performed a pyramid analysis of these loci without considering the effects of interactions among these QTLs and environmental influences. At the
germination stage, six recombinant types of three loci were detected within the population (Fig. 4a, Supplemental Table 3), and at the bud stage, a total of four recombinant types of three loci were detected (Fig. 4b, Supplemental Table 3). These results show that the corresponding phenotypic values increase with increasing pyramiding of favourable loci.

Discussion

A high-density genetic map can effectively improve the QTL mapping efficiency

The QTL mapping resolution depends on the marker density and the size of the confidence interval of the QTLs (Da et al. 2000, Visscher et al. 1996). In general, a gain in information as a result of an increased marker density results in reduced intervals; therefore, the application of additional markers is an effective way to increase the QTL mapping resolution (Liu et al. 2008). Compared with the previously published QTL map of low-temperature germinability, the genetic map of the population in this study was characterized by a higher marker density and a smaller average marker distance (Chen et al. 2006, Fujino 2004, Ji et al. 2008, Jiang et al. 2006, Wang et al. 2011). The physical interval of the five loci was only approximately 480 kb on average, and the smallest locus was approximately 21 kb (Tables 3, 4); only six genes were mapped to the Nipponbare reference genome. Thus, these factors collectively contribute to the fine mapping of these loci.

Different genetic loci control cold tolerance at the germination and bud stages in rice

Here, the same RIL population was used for the detection of QTLs associated with cold tolerance at the germination and bud stages. A total of five loci were detected; among these, two were unique to the germination stage,

Table 2. Pearson correlation matrix of eight traits in the RIL population

| Trait                | Germination rate-2 d (%) | Germination rate-4 d (%) | Germination rate-6 d (%) | Germination rate-8 d (%) | Germination index | Normal seedling rate (%) | Aberration rate (%) | Survival rate (%) |
|----------------------|--------------------------|--------------------------|--------------------------|--------------------------|-------------------|--------------------------|---------------------|------------------|
| Germination rate-2 d (%) | 0.519**                  | 0.207**                  | 0.080                    | 0.812**                  | 0.126*            | -0.032                   | 0.061               |
| Germination rate-4 d (%) | 0.491**                  | 0.610**                  | 0.327**                  | 0.862**                  | 0.088             | -0.062                   | 0.014               |
| Germination rate-6 d (%) | 0.240**                  | 0.642**                  | 0.769**                  | 0.657**                  | 0.054             | -0.032                   | 0.012               |
| Germination rate-8 d (%) | 0.100                    | 0.311**                  | 0.694**                  | 0.489**                  | 0.045             | 0.046                    | 0.063               |
| Normal seedling rate (%) | 0.685**                  | 0.897**                  | 0.773**                  | 0.573**                  | 0.123*            | -0.038                   | 0.055               |
| Aberration rate (%) | 0.172**                  | 0.169**                  | 0.131*                   | 0.079                    | 0.194**           | 0.027                    | 0.689**             |
| Survival rate (%) | -0.040                   | 0.044                    | 0.079                    | 0.103                    | 0.049             | 0.003                    | 0.743**             |
| (%)                  | 0.105                    | 0.157**                  | 0.151*                   | 0.127*                   | 0.179**           | 0.760**                  | 0.652**             |

* and ** indicate significance at the 0.05 and 0.01 levels, respectively.

Fig. 2. Number of bin markers and density for each chromosome within the RIL population. a, Number of bin markers; b, Bin marker density.
and two were unique to the bud stage. Interestingly, one common cold tolerance-related QTL was detected at both stages (Tables 3, 4, Fig. 3). However, the co-detection of QTLs at the germination and bud stages is rare. Gong et al. (2009) used a set of BIL populations and detected 19 QTLs at the germination stage, four QTLs at the bud stage and six QTLs at the seedling stage. Yang et al. (2014) detected one QTL at the bud stage and 17 QTLs at the germination stage for an RIL population derived from a *japonica* cross. There was no overlap in the QTLs obtained in these two studies. These results suggest that different genetic mechanisms control cold tolerance at the germination and bud stages in rice. Other research results have also shown that the cold tolerance of rice at different developmental stages might be controlled by different QTLs. In addition to the germination and bud stages, we focused further on the seedling and booting stages. Wang et al. (2011) detected two QTLs associated with cold tolerance at the germination stage and five

Table 3. QTLs associated with seed germination under cold stress in the RIL population of rice

| Loci  | QTL              | Env  | Chr. | Marker interval     | Physical interval (bp)   | LOD   | PVE (%) | ADD  | Known loci  |
|-------|------------------|------|------|---------------------|--------------------------|-------|---------|------|-------------|
| loci 1| qLTGR4d-9-1      | DS   | 9    | Block14586–Block14594 | 984,997–1,081,848         | 5.44  | 9.42    | 5.47 |             |
| loci 2| qLTGR4d-9-2      | WS   | 9    | Block15413–Block15423 | 11,962,250–12,143,018     | 3.13  | 5.13    | 5.22 | Ji et al. 2008 |
| qLTGR2d-9-1 | DS   | 9    | Block15413–Block15423 | 11,962,250–12,143,018     | 5.23  | 8.41    | 2.22 |             |
| qLTGI-9-1    | DS   | 9    | Block15413–Block15423 | 11,962,250–12,143,018     | 5.04  | 8.13    | 0.93 |             |
| loci 3| qLTGR2d-9-2      | WS   | 9    | Block15692–Block15747 | 15,950,479–17,727,187     | 3.66  | 5.96    | 1.25 |             |
| qLTGI-9-2    | WS   | 9    | Block15692–Block15747 | 15,950,479–17,727,187     | 4.28  | 6.94    | 0.89 |             |

*a* Environment: WS represents the wet season in 2017, and DS represents the dry season in 2017.  
*b* Chr., chromosome.  
*c* LOD, logarithm of odds.  
*d* ADD, additive effect; positive values indicate the superiority of H335.  
*e* PVE (%), phenotypic variation explained (%).

Fig. 3. All additive QTL positions on the high-density map. The blue patterns represent the QTLs detected during the WS, and the red patterns represent the QTLs detected during the DS. The text associated with different shapes corresponds to abbreviations of the different phenotypes. The circles represent the loci detected at both the germination and bud stages.
QTLs associated with cold tolerance at the seedling stage. Among the two sets of QTLs, only one QTL associated with cold tolerance at the germination stage, qLTSR-9-2, and one QTL associated with cold tolerance at the seedling stage, qLTSR-9-1, were mapped to the same region. Wang et al. (2019) detected two QTLs at the germination stage and one QTL at the seedling stage under cold stress, and there was no overlap in these QTLs. Using a mini-core collection of 174 Chinese rice accessions and 273 SSR markers, Pan et al. (2015) investigated cold tolerance at the germination and booting stages and detected 22 QTLs at the germination stage and 33 QTLs at the booting stage, and four of these QTLs overlapped. These results suggest that cold tolerance in rice might be controlled by different gene networks at different growth and development stages and that the expression of cold tolerance-related genes is spatiotemporal. However, a few QTLs associated with cold tolerance were detected at different stages, which suggests that there might be some overlap among the metabolic pathways associated with cold tolerance at different stages in rice. The same genetic loci were detected at different stages, which further demonstrates the reliability of our results. This type of cold tolerance-associated QTL is applicable to future studies of gene function. In addition, these same QTLs were detected repeatedly during the WS and DS. Notably, these di-genic interactions detected in the present study did not involve additive QTLs.

Here, we detected a total of five additive loci, and a pyramid analysis revealed that the accumulation of these loci can improve the corresponding traits even if epistatic effects are present (Fig. 4, Supplemental Table 4). The favourable alleles of the five loci originated from H335. However, some RIL offspring that exhibit an excellent cold-tolerant phenotype, such as G336, G367, G369, G411 and G417, were observed (Supplemental Table 5), and these RILs also harboured all five superior loci detected in the two periods. In addition, because the anaerobic germinability of rice is also very important for DSR, we also used this RIL population to analyse the QTLs associated with this trait. We identified valuable QTLs related to anaerobic germinability, and some RILs indeed harboured these QTLs (unpublished data). Combining the results of our low-temperature and anaerobic germinability analyses, we identified RILs that pyramid favourable alleles of these QTLs. These RILs could serve as donor parents of favourable alleles during the DSR breeding process.

Overall, we used a set of high-density genetic maps to analyse the genetic basis of cold tolerance during the germination and bud stages. A total of five additive loci and 30 pairs of epistatically interacting QTLs were detected. A pyramid analysis of five additive loci revealed that the accumulation of these loci could effectively improve the target traits. These loci are important donors for the breeding of cold-tolerant DSR and lay a foundation for future gene cloning efforts.

### Author Contribution Statement
TG, ZC, and JY designed the project, and JY performed all the experiments and wrote the manuscript. DL, HL, YL, MH and HW assisted in conducting the experiments and analysing the data. HW and ZC provided direction for the study and corrections to the manuscript. All the authors read and approved the final manuscript.
Identification of QTLs associated with the early germination stage under cold stress

**Table 5. Summary of the phenotypic effects of the five loci**

| Loci  | Traits                  | QTL\(^{a}\) | Env\(^{b}\) | Donor of positive allele | No. of RILs | Phenotypic value | Marker type 0 | Marker type 2 | Difference\(^{c}\) |
|-------|-------------------------|-------------|-------------|--------------------------|-------------|-----------------|--------------|--------------|----------------|
| loci 1 | Germination rate-2 d (%) | –           | WS          | 2                        | 135         | 1.32 ± 2.97     | 119          | 3.34 ± 5.81  | 2.02**        |
|       |                         | –           | DS          |                          |             | 3.62 ± 5.00     |              | 7.86 ± 8.48  | 4.24**        |
|       | Germination rate-4 d (%) | –           | WS          |                          |             | 39.63 ± 21.95   |              | 48.68 ± 23.16| 9.05**        |
|       |                         | –           | DS          |                          |             | 58.88 ± 18.06   |              | 68.75 ± 17.48| 9.86**        |
|       | Germination rate-6 d (%) | –           | WS          |                          |             | 85.65 ± 14.88   |              | 89.32 ± 12.46| 3.67*         |
|       |                         | –           | DS          |                          |             | 94.45 ± 6.43    |              | 93.83 ± 10.37| -0.63         |
|       | Germination rate-8 d (%) | –           | WS          |                          |             | 99.14 ± 2.13    |              | 98.47 ± 9.17 | 0.12          |
|       |                         | –           | DS          |                          |             | 97.68 ± 5.47    |              | 97.68 ± 5.47 | -0.67         |
|       | Germination index       | –           | WS          |                          |             | 19.41 ± 3.03    |              | 20.84 ± 3.81 | 1.43**        |
|       |                         | –           | DS          |                          |             | 22.14 ± 2.63    |              | 23.91 ± 3.94 | 1.77**        |
| loci 2 | Germination rate-2 d (%) | –           | WS          | 2                        | 123         | 1.22 ± 2.88     | 106          | 3.57 ± 6.06  | 2.36**        |
|       |                         | –           | DS          |                          |             | 3.21 ± 4.26     |              | 8.13 ± 8.73  | 4.92**        |
|       | Germination rate-4 d (%) | –           | WS          |                          |             | 38.99 ± 22.07   |              | 49.45 ± 23.40| 10.46**       |
|       |                         | –           | DS          |                          |             | 58.06 ± 17.94   |              | 68.48 ± 17.98| 10.42**       |
|       | Germination rate-6 d (%) | –           | WS          |                          |             | 85.42 ± 15.12   |              | 89.90 ± 12.48| 4.48*         |
|       |                         | –           | DS          |                          |             | 94.40 ± 6.46    |              | 93.56 ± 10.92| -0.84         |
|       | Germination rate-8 d (%) | –           | WS          |                          |             | 97.51 ± 5.69    |              | 97.83 ± 9.83 | 0.32          |
|       |                         | –           | DS          |                          |             | 99.08 ± 2.22    |              | 98.37 ± 9.71 | -0.70         |
|       | Germination index       | –           | WS          |                          |             | 19.30 ± 3.04    |              | 20.99 ± 3.89 | 1.69*         |
|       |                         | –           | DS          |                          |             | 21.96 ± 2.48    |              | 23.93 ± 4.09 | 1.98**        |
| loci 3 | Germination rate-2 d (%) | qLTGR2d-9-2 | WS          | 2                        | 122         | 1.18 ± 2.61     | 111          | 3.43 ± 5.93  | 2.25**        |
|       |                         | –           | DS          |                          |             | 3.68 ± 5.05     |              | 8.20 ± 9.45  | 4.53**        |
|       | Germination rate-4 d (%) | qLTGR4d-9-1 | WS          |                          |             | 38.84 ± 22.21   |              | 48.53 ± 21.90| 9.69**        |
|       |                         | –           | DS          |                          |             | 58.33 ± 18.53   |              | 68.11 ± 17.72| 9.78**        |
|       | Germination rate-6 d (%) | –           | WS          |                          |             | 84.41 ± 16.47   |              | 90.04 ± 10.89| 5.63**        |
|       |                         | –           | DS          |                          |             | 93.30 ± 10.73   |              | 94.55 ± 7.26 | 1.25          |
|       | Germination rate-8 d (%) | –           | WS          |                          |             | 96.56 ± 10.43   |              | 98.78 ± 2.64 | 2.22*         |
|       |                         | –           | DS          |                          |             | 98.17 ± 9.23    |              | 99.36 ± 1.23 | 1.19          |
|       | Germination index       | qLTGI-9-2   | WS          |                          |             | 19.12 ± 3.42    |              | 21.00 ± 3.29 | 1.88**        |
|       |                         | –           | DS          |                          |             | 21.94 ± 3.32    |              | 24.09 ± 3.61 | 2.15**        |
|       | Normal seedling rate (%)| –           | WS          |                          |             | 13.11 ± 20.75   |              | 22.74 ± 29.52| 9.63**        |
|       |                         | –           | DS          |                          |             | 10.23 ± 20.55   |              | 20.52 ± 29.96| 10.29**       |
|       | Aberration rate (%)     | –           | WS          |                          |             | 19.93 ± 20.17   |              | 26.01 ± 23.88| 6.08*         |
|       |                         | –           | DS          |                          |             | 17.80 ± 25.90   |              | 24.44 ± 31.86| 6.63          |
|       | Survival rate (%)       | qLTSR-9-2   | WS          |                          |             | 33.05 ± 31.02   |              | 48.75 ± 35.58| 15.71*        |
|       |                         | –           | DS          |                          |             | 28.04 ± 36.44   |              | 44.96 ± 41.72| 16.92**       |
| loci 4 | Normal seedling rate (%)| –           | WS          | 2                        | 132         | 12.32 ± 20.26   | 116          | 22.93 ± 29.27| 10.61**       |
|       | Aberration rate (%)     | –           | WS          |                          |             | 10.55 ± 19.98   |              | 20.65 ± 30.25| 10.10**       |
|       | Survival rate (%)       | qLTSR-9-1   | WS          |                          |             | 19.58 ± 20.59   |              | 25.15 ± 23.46| 5.57*         |
|       |                         | –           | DS          |                          |             | 17.50 ± 26.47   |              | 23.47 ± 30.95| 5.97          |
| lotti 5 | Normal seedling rate (%)| qLTNSR-9    | WS          | 2                        | 133         | 10.95 ± 18.39   | 118          | 22.77 ± 28.89| 11.82**       |
|       | Aberration rate (%)     | –           | WS          |                          |             | 19.43 ± 21.01   |              | 24.66 ± 23.23| 5.22          |
|       | Survival rate (%)       | qLTSR-9-2   | DS          |                          |             | 17.43 ± 26.68   |              | 22.93 ± 30.82| 5.50          |
|       |                         | –           | WS          |                          |             | 30.38 ± 30.18   |              | 47.43 ± 35.10| 17.05**       |

\(^{a}\) QTL, “-” indicates that no QTL was detected during the season.

\(^{b}\) Environment: WS represents the wet season in 2017, and DS represents the dry season in 2017.

\(^{c}\) Phenotype of an elite allele minus that of a nonelite allele, and ** indicates significance at the 0.01 level.
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