



Full Length Article

QTL Mapping for Cold Tolerance at Early Seedling Stage in Dongxiang Wild Rice (*Oryza rufipogon* Griff.) under Severe Cold Stress

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Abstract

Rice (*Oryza sativa* L.) is highly sensitive to temperature and severely vulnerable to low temperature in temperate and high-altitude rice growing areas. Cold tolerance at the early seedling stage is a favorable trait for ensuring uniform seedling establishment and performance during early-season planting in double cropping rice cultivation. Detection of quantitative trait loci conferring cold tolerance at early seedling stage was conducted using backcross recombinant inbred lines derived from an interspecific cross between *Oryza sativa* and *O. rufipogon* and three cold treatments. In total, ten quantitative trait loci conferring early seedling cold tolerance were detected in all treatments and explained phenotypic variation ranging from 4.1% to 45.1%, including three in treatment T1, five in T2, and six in T3. Four of ten quantitative trait loci were detected in two treatments. Eight of the ten quantitative trait loci had the favorable alleles originated from *O. rufipogon*. The quantitative trait loci identified in this report might be used for molecular breeding and improvement of seedling cold tolerance in rice using marker assisted quantitative trait loci pyramiding. © 2019 Friends Science Publishers

Keywords: Common wild rice (*Oryza rufipogon* Griff.); Low temperature tolerance; Early growth stage; Quantitative trait loci

Introduction

Low temperature is a pivotal factor affecting the geographic distribution, growth, productivity, and grain quality of field crops (Farooq *et al.*, 2009, 2017), which also affects their planting season. As an important staple food crop for more than 50% the world's population, rice (*Oryza sativa* L.) inhabits tropical and subtropical areas having the optimum growth temperature range between 25°C and 30°C (Lu *et al.*, 2014). Therefore, most rice cultivars are extremely vulnerable to the cold injury. Cold stress is one of the main environmental stresses that can occur at any developmental stage between germination and maturity, which leads to a decrease of rice yield and quality (Ye *et al.*, 2009; Cruz *et al.*, 2013; Arshad *et al.*, 2017). During the early spring in temperate and high-altitude rice growing areas, rice seedlings can frequently suffer from cold stress, which can result in the stunted growth habit, leaf withering and discoloration, and eventually result in the heterogeneous maturation (Kim *et al.*, 2014). Owing to its dominant advantages such as lower irrigation demands and labor inputs, direct-seeded rice has gradually replaced conventional transplanted rice during rice cultivation (Farooq *et al.*, 2011; Peng, 2014; Sandhu *et al.*, 2015), which is becoming more and more prevalent in many Asian countries including China. However, in direct-seeded

rice cultivation, the soil and water temperature of the paddy field is frequently below 15°C during the early-season sowing period, while the optimum seed germination and seedling growth temperature is between 25°C and 35°C (Wang *et al.*, 2018). Cold tolerance at the early seedling stage, therefore, is of great importance for ensuring uniform seedling establishment and stable rice production in the direct-seeded early season rice (Cruz and Milach, 2004). On the other hand, cold tolerance at the early growth stage in rice can extend the growing season through earlier growth during spring in temperate or subtropical zone. Therefore, it is very important to understand the mechanisms that underlie cold tolerance and to develop seedling stage cold-tolerant cultivars for rice production.

Over the past few decades, considerable breeding efforts have been paid to improve the tolerance of rice to cold stress. Despite this, however, not much progress has been made in the genetic improvement of cold tolerance because cold tolerance in rice is a very complex trait that is controlled by quantitative trait loci (QTLs) (Zhang *et al.*, 2014a) and the limited rice germplasms with strong cold tolerance are available. Thus, the breeding and improvement of cold tolerance in rice using traditional breeding technique is not desirable in practice. However, detection of QTLs conferring cold tolerance can provide an effective and promising

approach to understand genetic mechanisms of this adaptation in rice, and marker-assisted QTL pyramiding would facilitate the improvement of cold tolerance in rice. Up to date, over 250 cold tolerant QTLs in rice at different growth stages have been mapped on all 12 chromosomes using various populations derived from interspecific and/or intraspecific crosses and evaluation criteria (Koseki *et al.*, 2010; Wang *et al.*, 2011; Kim *et al.*, 2014; Zhang *et al.*, 2014b; Mao *et al.*, 2015; Pan *et al.*, 2015; Zhu *et al.*, 2015; Luo *et al.*, 2016; Zhao *et al.*, 2017; Yang *et al.*, 2018). Some of those QTLs were fine-mapped, cloned and characterized, including five cold tolerant QTLs, *COLD1*, *qCTS-9*, *qLTG3-1*, *HANI* and *LTG1*, for the early growth stage (Fujino *et al.*, 2008; Lu *et al.*, 2014; Ma *et al.*, 2015; Zhao *et al.*, 2017; Mao *et al.*, 2019) and two QTLs, *Ctb1* and *CTB4a*, for the reproductive stage (Saito *et al.*, 2010; Zhang *et al.*, 2017). For a better understanding of cold tolerance in rice, it is essential to identify additional QTLs/genes in rice, especially in wild relatives.

Dongxiang common wild rice (*Oryza rufipogon* Griff., referred to as DCWR) is the northernmost rice worldwide, which is located in Dongxiang county, Jiangxi province. This species is the progenitor of the cultivated Asian rice and belongs to a national second-class protected plant in China (Xie *et al.*, 2010). So far, numerous QTLs have been identified and even cloned for various important traits in DCWR, including cold tolerance (Xiao *et al.*, 2014, 2015; Mao *et al.*, 2015; Luo *et al.*, 2016), fertility restoration (Hu *et al.*, 2016), cytoplasmic male sterility (Xie *et al.*, 2018), and overwintering (Liang *et al.*, 2018). Of these, the strong cold tolerance is the most remarkable trait, promoting DCWR to overwinter safely at low temperatures of minus 12.8 degrees (Mao *et al.*, 2015). Therefore, DCWR has been a very important and valuable germplasm for studying the mechanisms of rice cold tolerance. Previous QTL mapping studies on cold tolerance of DCWR have focused on the seedling and booting stage. As for seedling stage, over 56 cold tolerant QTLs were detected on all the 12 chromosomes using various bi-parental populations derived from DCWR, assessment methods and parameters (Liu *et al.*, 2013; Xiao *et al.*, 2014, 2015; Mao *et al.*, 2015; Luo *et al.*, 2016), and 92.85% (52/56) of these QTLs had beneficial alleles contributed by DCWR. Although these candidate QTLs that conferred cold tolerance to DCWR in the bi-parental populations are known, this information is still insufficient to reveal the genetic mechanisms on the strong cold tolerance of DCWR in the northernmost habitat, compared with rice cultivars and other wild species, accordingly, which limited its extensive applications to facilitate rice cold tolerance breeding.

In the present study, detection of the QTLs conferring cold tolerance at the early seedling stage was conducted to using a backcross-inbred line population derived from the interspecific cross between a rice cultivar Xieqingzao B and an accession of DCWR. QTL analysis derived from DCWR will provide additional or novel alleles to improve cold

tolerance of rice and genetic information to understand the molecular mechanism underlying this adaptation.

Materials and Methods

Plant Materials

A total of 202 backcross inbred lines (BILs, BC₁F₅), derived from the interspecific backcross between Xieqingzao B (XB) and DCWR by the single-seed descent (Chen *et al.*, 2006), was used for QTL detection. The recurrent parent XB, a cold-susceptible *indica* cultivar, is the maintainer line of Xieqingzao A belonging to dwarf-wild-abortive type cytoplasmic male sterility (*O. sativa* ssp. *indica*), and the donor parent DCWR, a cold-tolerant wild relative, is an *O. rufipogon* accession collected from *in-situ* conservation populations in Dongxiang county, Jiangxi Province, which is no longer available.

Phenotypic Evaluation of Cold Tolerance

A randomized complete block design was performed to evaluate cold tolerance of the BILs in a temperature-controlled phytotron growth chamber. 50 seeds of each BIL and the recurrent parent XB were treated in a drying oven at 45°C for 48 h for breaking dormancy. After surface-sterilization in 0.6% sodium hypochlorite solution (Murashige and Skoog, 1962), rice seeds were washed with distilled water four times. All the seeds were placed on Whatman filter paper saturated with distilled water in 90 × 10 mm Petri dishes and incubated at 30°C for 3 d. After germination, the seedlings were grown in the 90 × 10 mm Petri dishes containing the Murashige and Skoog solution. At the one-leaf stage, 40 strong and healthy seedlings of each line were subjected to the cold treatment at 3°C for 72 h. After recovery growth for seven days, cold tolerance was evaluated based on the percentage of the seedling mortality (SM). The seedlings were regarded as dead if their leaves were completely withered, whereas those exhibiting normal growth with green leaves were regarded as surviving. The percentage of the SM was calculated as the following formulas: SM% = dead seedlings/total seedlings × 100. The recovery growth experiments were conducted in the growth chamber with a 16/8 h light/dark photoperiod (32°C/28°C) and 70% relative humidity. The evaluation of cold tolerance was performed with two replications under three cold treatments (T1, T2, and T3). The mean data of the SM over two replications were used for data analysis.

Data Analysis and QTL Mapping

The genetic map in this report was previously constructed by Chen *et al.* (2006) and Huang *et al.* (2008), respectively, which consisted of 149 DNA markers including 108 simple sequence repeats and 41 restriction fragment length

polymorphism markers. It covered a total genetic distance of 1306.4 cM, with an average distance of 9.5 cM between adjoining markers.

The mean data of the seedling mortality over two replications were used for QTL mapping. QTL detection was conducted using the Composite Interval Mapping (CIM) approach of the Windows QTL Cartographer ver. 2.5 (Wang *et al.*, 2012). CIM model 6, backward and forward regression at a probability threshold of 0.01, a filtration window size of 10 cM and a walking speed of 1 cM were chosen for the genome scan. A logarithm of the odds (LOD) significance threshold of 2.0 and above was used to determine a putative QTL. The QTLs were designated following the nomenclature proposed by McCouch and CGSNL (2008).

The result of phenotypic statistics including minimum, maximum, mean, standard deviation (SD), skewness, kurtosis, and coefficient of variation (CV) were calculated by the DSum function of the Windows QTL Cartographer 2.5 (Wang *et al.*, 2012). Pearson correlation coefficient between the cold treatments was calculated using the Pearson function of the Microsoft Excel 2007.

Results

Phenotypic Performance of Cold Tolerance

Table 1 presents the descriptive statistics of SM of the BILs in three cold treatments. Among three treatments, CV of SM in the treatment T1 is the highest (0.50), and that of SM in the treatment T3 is the lowest (0.41). The SM exhibited wide phenotypic variations with continuous distribution in the BILs in all three cold treatments (T1, T2, and T3), suggesting that cold tolerance is controlled by multigenes. The mean SM values of the BILs in the treatments T1, T2 and T3 were 59.91%, 68.31%, and 64.13%, respectively, and much less than those of their corresponding parent XB. Based on the Pearson correlation analysis, strong positive and highly significant correlations ($P < 0.01$) were found for the SM value between the treatments T1 and T2, T2 and T3, and T1 and T3, with coefficients of 0.579, 0.886, and 0.893, respectively.

QTL Analysis

In total, ten QTLs with a LOD score more than 2.0 were detected for early seedling cold tolerance, including three QTLs for the SM in the treatment T1, five in the treatment T2, and six in the treatment T3 (Table 2 and Fig. 1). These were distributed on the chromosomes 2, 4, 5, 7, 8 and 9 (Fig. 1) and the phenotypic variance (R^2) explained by an individual QTL ranged 4.1–45.1%.

In the treatment T1, three QTLs for the SM were mapped on chromosomes 2, 4, and 8, respectively, and explained ranging from 5.3% to 6.5% of the phenotypic variance. Two of the QTLs, *qCTS4* and *qCTS8*, had the trait-enhancing alleles from XB, which increased the seedling mortality by 12.81% and 9.28%, respectively. The remaining

QTL *qCTS2.1* had a LOD score of 3.25 and the highest R^2 (6.5%), with the enhancing allele derived from DCWR.

In the treatment T2, five QTLs for the SM were detected on the chromosomes 5, 7 and 9. The QTL *qCTS9.1* was the only major-effect QTL detected, having a LOD score of 5.58 and 41.5% of R^2 . The remaining four QTLs explained 6.3%–8.0% of the total phenotypic variance. The trait-enhancing alleles at the five loci were all derived from DCWR, and caused a decrease of 10.10%–13.92% of the SM in seedlings.

In the treatment T3, six QTLs for the SM were detected, and they explained from 4.1% to 6.2% of R^2 . Among these, *qCTS4* with a LOD score of 2.40 explained 6.2% of the phenotypic variance, and the XB allele at this locus increased SM by 11.54%. For the other five QTLs, the trait-enhancing alleles were all derived from DCWR.

Discussion

Cold temperature stress at the early seedling stage is one of the major restrictions affecting the seedling establishment and performance of rice in temperate and high-altitude rice growing areas. Therefore, cold tolerance at the early seedling stage is the highest priority in rice breeding programs, which could be widely utilized in the direct-seeded rice cultivation.

DCWR possesses an exceedingly strong innate tolerance to low temperature, and it is invaluable to improve cold tolerance in rice breeding. Therefore, DCWR has been made considerable efforts in germplasm to improve cold tolerance in rice. However, the mechanisms on the adaption to the cold temperatures of the northernmost habitat remain unclear in DCWR. On the other hand, the cold stress intensity (in terms of the treatment temperature and/or duration) play pivotal role in the accurate and effective cold tolerance evaluation. In present study, cold treatment of more severe stress intensity (3°C for 72 h) was employed to evaluate the cold tolerance of the BILs derived from DCWR, compared to previous studies (Mao *et al.*, 2015; Xiao *et al.*, 2014, 2015; Luo *et al.*, 2016). The seedling mortality of the BILs showed a continuous distribution, suggesting that the intensity of the cold stress (3°C for 72 h) was suitable for evaluating cold tolerance at the early seedling stage and QTL detection. As a result, a total of ten QTLs were found to be associated with this trait. Four QTLs (*qCTS4*, *qCTS5.1*, *qCTS9.1*, and *qCTS9.2*) were stably detected in two of three cold treatments. Notably, the *O. rufipogon*-derived alleles at the eight loci (80%) could improve cold tolerance of rice seedlings in the XB background, which may explain the strong cold tolerance of DCWR. On the other hand, two of the ten cold-tolerant QTL alleles derived from cold-sensitive parent XB, suggesting that *indica* also harbor some cold tolerant alleles, which coincided with the results reported by Zhang *et al.* (2012) and Zhu *et al.* (2015). Moreover, the QTL analysis results revealed that cold tolerance at the early seedling stage

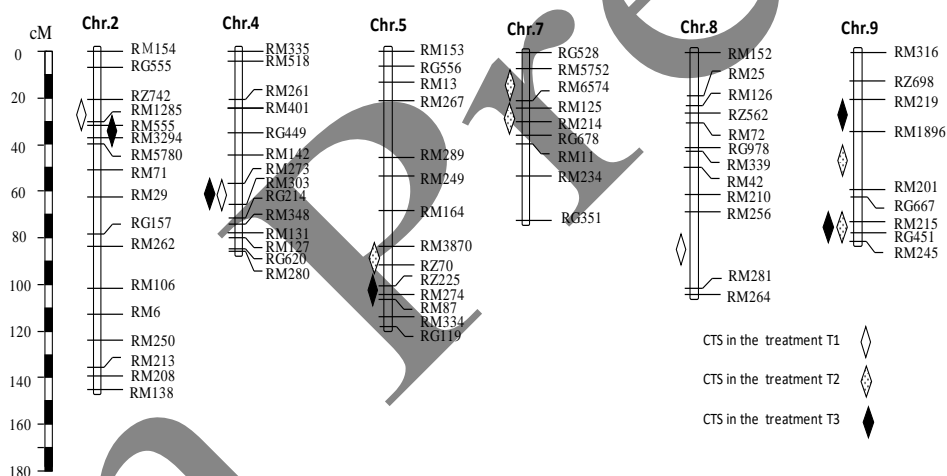
Table 1: Phenotypic performance of seedling mortality (%) in a BIL population of Xieqingzao B (XB)//XB/Dongxiang wild rice

Trait	Treatment	Mean	SD	CV	Range	Skewness	Kurtosis	XB
SM	T1	59.91	29.87	0.50	0.00-100.00	-0.34	-1.06	92.8
	T2	68.31	29.14	0.43	0.00-100.00	-0.70	-0.62	97.6
	T3	64.13	26.19	0.41	0.00-100.00	-0.37	-0.92	95.7

Table 2: QTLs for cold tolerance at the early seedling stage detected in the BIL population of XB//XB/Dongxiang wild rice

Treatment	QTL	Interval	LOD	A^a	R^2	Previous reports
T1	<i>qCTS2</i>	RZ742-M1285	3.25	-9.14	6.5	Yang <i>et al.</i> , 2013; Ma <i>et al.</i> , 2015
	<i>qCTS4</i>	RM273-M303	2.48	12.81	5.3	
	<i>qCTS8</i>	RM256-M281	2.63	9.28	5.5	
T2	<i>qCTS5.1</i>	RM3870-RZ70	3.38	-10.10	6.3	Liu <i>et al.</i> , 2013
	<i>qCTS7.1</i>	RM5752-M6574	2.29	-12.83	8.0	Mao <i>et al.</i> , 2015
	<i>qCTS7.2</i>	RM125-M214	2.71	-13.92	6.4	Liu <i>et al.</i> , 2013
	<i>qCTS9.1</i>	RM1896-M201	5.58	-24.08	41.5	Xiao <i>et al.</i> , 2014; Zhang <i>et al.</i> , 2014b; Zhao <i>et al.</i> , 2017
	<i>qCTS9.2</i>	RG451-RM245	3.62	-10.76	7.9	
T3	<i>qCTS2</i>	RM555-M3294	2.88	-8.78	6.5	Liu <i>et al.</i> , 2013 Yang <i>et al.</i> , 2013 Xiao <i>et al.</i> , 2014; Zhang <i>et al.</i> , 2014b; Zhao <i>et al.</i> , 2017
	<i>qCTS4</i>	RM273-M303	2.40	11.54	6.2	
	<i>qCTS5.1</i>	RM3870-RZ70	3.18	-8.76	6.0	
	<i>qCTS5.2</i>	RZ255-RM274	2.11	-7.04	4.1	
	<i>qCTS9.1</i>	RM219-M1896	2.25	-8.28	5.4	
	<i>qCTS9.2</i>	RG451-RM245	2.53	-8.15	5.6	

^a A indicates that an additive effect of replacing a Xieqingzao B allele by a Dongxiang wild rice allele; R^2 indicates that the proportion of phenotypic variance explained by the QTL effect

**Fig. 1:** Chromosomal position of the QTL for cold tolerance at early seedling stage

was mainly controlled by one major QTL with phenotypic variance explained (PVE) of 41.5%, and multiple minor loci with PVE of less than 10% individually, which is consistent with a previous study (Koseki *et al.*, 2010).

To assess the QTL reliability in this report, the physical positions of the QTLs detected were compared to those of the QTLs for the same trait at the seedling stage identified in previous studies using the Gramene annotated nipponbare sequence 2009 map (Gramene, 2013). As a result, six of ten QTLs overlapped with the published QTLs. *qCTS5.1* and *qCTS5.2* co-located with the QTL for seedling survival rate in previous studies (Liu *et al.*, 2013; Yang *et al.*, 2013). *qCTS7.1* and *qCTS7.2* overlapped with the QTL for seedling survival rate detected by Mao *et al.* (2015) and Liu *et al.* (2013), respectively. *qCTS8* corresponded the QTL *qCTS8*

identified by Yang *et al.* (2013) and *COLD2* identified by Ma *et al.* (2015), respectively. *qCTS9.1* coincided with a QTL identified in the three studies (Xiao *et al.*, 2014; Zhang *et al.*, 2014b; Zhao *et al.*, 2017). Furthermore, two QTL regions, *qCTS7.2* and *qCTS9.1*, contained a candidate gene *LOC_Os07g22494* and a functional gene *Os09g0410300*, respectively, both related with cold tolerance (Liu *et al.*, 2013; Zhao *et al.*, 2017). Results from QTL comparison detected in different environments and genetic backgrounds showed accuracy and consistency of QTLs identified for seedling survival rate or/and seedling mortality. In addition, all these QTLs were detected in different studies, providing good candidates for the allelic relationship studies, QTL fine-mapping and cloning.

On the other hand, the remaining four cold tolerance

QTLs (*qCTS2.1*, *qCTS2.2*, *qCTS4*, and *qCTS9.2*) were firstly detected in the present study. Furthermore, the enhancing alleles at three novel loci (*qCTS2.1*, *qCTS2.2* and *qCTS9.2*) are likely to be the *O. rufipogon*-specific alleles existing in DCWR, which requires further confirmation. Ma *et al.* (2015) reported that the SNP2 in *COLD1*, derived from *O. rufipogon*, conferred chilling tolerance to *japonica* rice. At SNP2 site, XB had the nucleotide T, whereas DCWR had A, which is associated with strong artificial selection during domestication. Similarly, the favorable alleles were found to derive from wild rice, contrasting with previous studies showing that these originated from cultivated rice. More evidence is needed to investigate whether this difference results from ecological adaptation or artificial selection in the domestication process.

The XB//XB/DCWR BILs were previously employed to detect QTL for yield components (Huang *et al.*, 2008) and resistance to the whitebacked planthopper (Chen *et al.*, 2010), respectively. Three QTLs identified by Huang *et al.* (2008), *qTGWT-8*, *qTNSP-9*, and *qNFGP-9*, were located in the corresponding regions of *qCTS8*, *qCTS9.1*, and *qCTS9.2*, respectively. Regarding the second trait, among the three QTLs detected by Chen *et al.* (2010), *qWph5* and *qWph9* were located in the regions for *qCTS5.1* and *qCTS9.2*, respectively, and the favorable *O. rufipogon* alleles decreased the seedling mortality in both abiotic and biotic stress. It is necessary to further ascertain the allelic relationship between the QTLs for cold tolerance, grain yield and resistance to the whitebacked planthopper located in the same chromosomal region.

Jiangxi province is a traditional area for double-season rice cropping in China. In the early rice season in Jiangxi province, growers would anticipate sowing date as early as possible to extend the rice-growing season and, in consequence, to increase grain yield. However, the planting of early-season rice usually delayed by cold spells in early spring, which subsequently delay the planting of the following late-season rice, and thus ultimately affects flowering and grain filling when low temperature occurs (middle or late September). From this perspective, the development of early-season cold tolerant rice is important for the double-cropping rice areas, especially for direct-seeded cultivation. Therefore, the molecular markers linked to the QTLs and the favorable *O. rufipogon* alleles detected in present report could be used to develop early-season *indica* rice cultivars with cold tolerance at the early seedling stage for direct-seeded cultivation in double-cropping rice area.

Conclusion

The early seedling cold tolerance is a favorable characteristic for ensuring uniform seedling establishment and stabilizing grain yield in temperate and high-altitude rice growing regions, which is a quantitative trait controlled by multiple genes. Further studies are needed to fine map and clone these novel QTLs conferring cold tolerance at the

early seedling stage in the future, and the favorable *O. rufipogon* alleles identified could be utilized to improve seedling cold tolerance in rice using marker-assisted QTL pyramiding.

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