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ORIGINAL PAPER

Multiple cold resistance loci confer the high cold tolerance adaptation of Dongxiang wild rice (*Oryza rufipogon*) to its high-latitude habitat

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Abstract

Key message **Dongxiang wild rice is phylogenetically close to *temperate japonica* and contains multiple cold resistance loci conferring its adaptation to high-latitude habitat.**

Abstract Understanding the nature of adaptation in wild populations will benefit crop breeding in the development of climate-resilient crop varieties. Dongxiang wild rice (DXWR), the northernmost common wild rice known, possesses a high degree of cold tolerance and can survive overwintering in its native habitat. However, to date, it is still unclear how DXWR evolved to cope with low-temperature environment, resulting in limited application of DXWR in rice breeding programs. In this study, we carried out both QTL mapping and phylogenetic analysis to discern the genetic mechanism underlying the strong cold resistance. Through a combination of interval mapping and single locus analysis in two genetic populations, at least 13 QTLs for seedling cold tolerance were identified in DXWR. A phylogenetic study using both genome-wide InDel markers and markers associated with cold tolerance loci reveals that DXWR belongs to the Or-III group, which is most closely related to cold-tolerant *Japonica* rice rather than to the *Indica* cultivars that are predominant in the habitat where DXWR grows. Our study paves the way toward an understanding of the nature of adaptation to a northern habitat in *O. rufipogon*. The QTLs identified in DXWR in this study will be useful for molecular breeding of cold-tolerant rice.

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Introduction

Rice (*Oryza sativa* L.) is a staple crop that is sensitive to low temperatures, which can retard development and reduce the grain setting rate. With the intensification of global climate change, cold stress has already become one of the major environmental adversities that cause yield losses in rice. Moreover, with more and more farmers leaving their farms for jobs in the cities, direct sowing of the seed has replaced hand transplanting of seedling as a major choice in rice culture due to the shortage of farm labor. As a consequence, cold resistance has become recognized as a highly desirable trait for direct sowing of early rice in the double-cropping rice regions, because it is impossible to protect directly sown rice seedlings from cold stress in fields such as seedbeds. Thus, the study of cold tolerance in rice is of great significance for the stability of food production.

Temperature, especially lower temperatures, is one of the main environmental factors driving plant evolution. Temperature determines the geographic distribution and divergence of plant species. Rice has evolved as two distinct subspecies, *Indica* and *Japonica*. Generally speaking,

Indica rice cultivars are cold sensitive and are distributed mainly in tropical and subtropical regions, while *Japonica* cultivars are more tolerant to low temperatures, and have adapted to temperate climates or higher altitudes. Cold tolerance has been one of the important indexes to distinguish the two rice subspecies (Oka 1958). High-throughput re-sequencing and comparative analyses of numerous cultivated rice genomes have also shown that loci that differentiate the *Indica* and *Japonica* subspecies are close to regions in the genome that contain QTLs associated with cold tolerance, such as *qCTS12* (Andaya and Tai 2006; Huang et al. 2011, 2012). As the progenitor of cultivated rice, common wild rice can also be divided into three subpopulations, designated Or-I, Or-II, and Or-III. The first and last of these are regarded as being the progenitors of the subspecies of cultivated *Indica* and *Japonica* rice, respectively (Huang et al. 2012). However, a system using cold resistance as an index to sub-classify common wild rice has not been established. Elucidating the phylogenetics of wild rice and its relationship with temperature resilience will benefit both our understanding of the mechanisms of natural adaption, and genetic improvement using common wild rice as a valuable gene resource (Khush 1997; Zhang et al. 2006; Geng et al. 2008; Thalapati et al. 2012; Yang et al. 2012).

Dongxiang wild rice (DXWR; *Oryza rufipogon*), a Chinese type of wild rice grown in Jiangxi Province (116°36′E, 28°14′N), is the most northerly wild rice grown in the world. DXWR possesses extremely high tolerance to cold stress and is able to safely survive winter temperatures as low as −12.8 °C (He et al. 1996). Thus, it may be an invaluable genetic resource for improving cold tolerance in cultivated rice. To date, there have been several reports describing the genetic analysis or molecular mechanisms underlying cold tolerance in DXWR. For example, several QTLs for cold tolerance at the seedling, booting, or flowering stages have been mapped to chromosomes 1, 3, 4, 6, 8, 10 and 11 (Chen et al. 2002; Liu et al. 2003; Xia et al. 2010; Zuo et al. 2012; Xiao et al. 2014). Also *OrbHLH001*, which encodes an ICE1-like protein, was the only gene isolated from DXWR that enhanced cold tolerance when expressed in transgenic *Arabidopsis* (Li et al. 2010). However, there was no evidence showing that this gene could explain any cold tolerance QTLs of DXWR. Although these invaluable studies identified several QTLs that conferred cold tolerance to DXWR compared to the cultivated parent in the genetic populations, it was still difficult to explain the genetic basis of its high degree of cold resistance, compared to other wild rice species and cultivars, by which it adapted to low temperatures in such a northern habitat. There are two possible reasons for our inability to understand the strong cold tolerance of DXWR. The first is the open question regarding its phylogenetic relationship to the two subspecies of cultivated rice. All of the cultivated parents of these mapping populations were cold-sensitive *Indica* rice cultivars. The identified QTLs may then be QTLs common to both DXWR and *Japonica* rice rather than the QTLs unique to DXWR. Second, because all of the QTLs above were identified using the older generation of markers such as simple sequence repeats (SSRs), which were sparsely distributed in many regions of rice genome; it is not possible to obtain precise and complete information about the numbers and locations of cold-tolerant QTLs from DXWR. These ambiguous results hindered our understanding of the natural adaption of DXWR and limited its use in molecular breeding of cold-tolerant rice. To date, there is only one cold-tolerant commercial rice variety, Dongye 1 (DY1), derived from the offspring of a hybrid of DXWR and rice cultivar 0298 (Chen et al. 2007).

The development of next-generation sequencing (NGS) technologies has made it practical to use DNA sequencing to directly obtain single nucleotide polymorphism (SNP) markers for population genotyping (Schuster 2008). Using a combination of NGS and reduced representation libraries (RRL), specific-locus amplified fragment sequencing (SLAF-Seq) has been developed (Sun et al. 2013). SLAF-Seq is an ideal tool for large-scale genotyping in QTL mapping or gene discovery with high resolution (Zhang et al. 2013; Liu et al. 2014; Qi et al. 2014). In this study, we employ two genetic populations based on DXWR; the first is composed of backcross inbred lines (BILs) for interval mapping of cold-tolerant QTLs on a high-density SLAF-Seq genetic map, and the second is a population of recombinant inbred lines (RILs) for confirmation of QTLs by single marker analysis (SMA). The mapped QTLs are determined in DY1, the highly cold-tolerant cultivar derived from DXWR mentioned above (Chen et al. 2007), to determine how many cold-tolerant QTLs were pyramided in its cold-tolerant progeny. Then, using markers linked to QTLs and genome-wide InDel markers, we analyzed the phylogenetic relationship of DXWR with numerous wild rice accessions and cultivated rice lines.

Materials and methods

Plant materials

As shown in the Fig. S1, two genetic populations were employed to study the genetic basis of cold tolerance in DXWR. The population for SLAF-seq contained 94 BILs (BC_1F_7) derived from a hybrid between DXWR and Xieqingzao B (XQZB, *Indica*). The population for SMA comprises 146 RILs (F_7), which were developed by single seed descent from a cross between DXWR and 1504 (*Indica*). Both XQZB and 1504 are low-temperature sensitive

double-cropping early rice cultivars. The progeny derived from hybridization with DXWR were designated DX-BILs and DO-RILs, respectively. DY1, a very cold-tolerant variety, was developed through several years of breeding and selection under cold stress conditions from the progeny of a hybrid between DXWR and 0298 (*Japonica*) (Chen et al. 2007). To perform phylogenetic analysis of DXWR, we sampled 40 other common wild rice accessions distributed around the world plus 39 cultivated rice accessions which had been divided into five ecotypes (Garris et al. 2005). Most of this rice germplasm was kindly provided by the International Rice Researching Institute (IRRI) in the Philippines, and are listed in the Supplement Spreadsheets 1 and 2.

Evaluation of low-temperature tolerance

Germinated rice seeds were grown hydroponically in half-strength of Hoagland's solution in a plant growth chamber (CU-41L4, Percival Scientific Inc. USA) at 28/25 °C day/night with a 13-h photoperiod (220 mol/m^2/s) for 7 days. The 7-day-old seedlings were then treated with low temperature (8 °C) for 2–16 days under the same photoperiod and light intensity. The seedlings were then returned to normal temperatures for a 5-day recovery period. Lastly, their cold tolerance was scored according to their status after cold treatments.

Two indices, cold resistance index (CRI) and percent of survival (SUR) were adopted to quantify the seedling cold tolerance. According to the performance after treatment, each plant was classified into one of the four grades of CRI: (1) 0—entire plant wilted and dead; (2) 1—plants had one green leaf (more than 2/3 of the leaf was green); (3) 2—plants had two green leaves; (4) 3—plants had two green leaves and also a new leaf emerging (Fig. S2). The average of CRI of all seedlings was referred to the CRI of each line in each treatment. For the SUR, the percentage of seedling survived in each treatment was recorded for every line. All the treatments were repeated at least three times, and the average of repeats was used to characterize the cold tolerance. To phenotype the cultivated accessions and the progenies of the genetic populations, a total of 30–50 healthy plants were cold treated in each independent repeat. Due to limited number and low ratio of germination of wild rice seeds, only ~5 seedlings were finally phenotyped in each of three repeats and only CRI was used to analyze the Pearson correlation among CRI, latitude and genetic distance in wild rice populations.

SLAF-seq and InDel/SNP marker development

Genomic DNA was extracted using the cetyl-trimethyl ammonium bromide method with minor modifications (Murray and Thompson 1980). DNA concentration was measured with a NanoDrop-2000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and DNA quality was estimated by electrophoresis in 0.8 % agarose gels.

Genotyping of the DX-BILs was performed using SLAF-seq as described by Sun et al. (2013) with a few modifications. In brief, the genomic DNA was initially digested with MseI, and the resulting fragments were ligated to sequencing connectors. DNA fragments of lengths 330–380 base pairs (bp) were selected for pair-end sequencing using an Illumina high-throughput sequencing platform (Illumina Inc.; San Diego, CA, USA). Thereafter, SLAF reads with clear indexes were clustered based on sequence similarity, and sequences with over 90 % identity were grouped to a single SLAF locus. Each SLAF with an SNP/InDel was referred to as a polymorphic marker, and each progeny plant was then genotyped for these polymorphic SLAFs based on the sequences from the two parents.

High-density linkage map construction and QTL analysis

Using high-throughput SLAF-seq data, a high-density linkage map for the DX-BIL population was constructed using the HighMap method. The mapping process involved the following four steps: (1) the SLAF markers were partitioned into linkage groups using a single-linkage clustering algorithm based on a pairwise modified independent LOD score for the recombination frequency; (2) using a combination of Gibbs sampling, spatial sampling, and the simulated annealing algorithm, the ordering module sequenced SLAF markers and estimated linkage distances; (3) using the k-nearest neighbor algorithm, the false singletons were eliminated from the data based on parental contribution of genotypes. To order markers correctly, the ordering and error correction processes were performed iteratively; (4) heat maps and haplotype maps were constructed to evaluate map quality (Liu et al. 2014).

The QTL analysis was performed by interval mapping with QTLMap6 packet (http://www.kyazma.nl/index.php/mc.MapQTL/). When the LOD score, the logarithm of the likelihood ratio of QTL presence to absence, exceeded the significance threshold of 3.0, QTLs for cold tolerance were determined on the linkage groups. The position on the linkage group with the largest LOD score is the estimated position of the QTL on the map. The 95 % confidence intervals for QTLs were determined by the left and right points having an LOD value of 2 less than the peak point (van Ooijen et al. 2002). To confirm the QTLs detected above, single marker analysis was performed in the DO-RIL population using the software QTL Cartographer 2.5 (Wang et al. 2012).

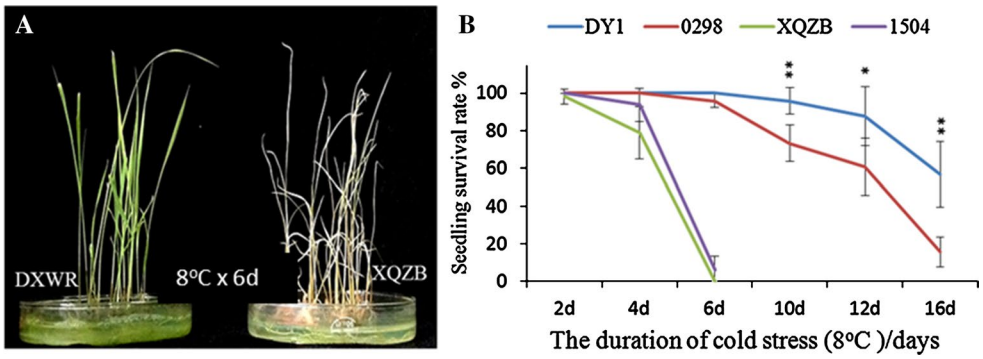

Fig. 1 Cold tolerance of DXWR, its derivative DY1, and the cultivated parents XQZB and 1504. **a** Performance of 7-day-old seedlings of DXWR and XQZB after 6 days of cold treatment at 8 °C, followed by 5 days of recovery at normal growth temperatures. **b** Performance of 7-day-old seedlings of DY1, 0298, 1504, and XQZB after 2–16 days of cold treatment at 8 °C, followed by 5 days of recovery at normal growth temperatures. Each rice variety was treated four times in independent experiments and the *bars* represent the standard deviation. *One* (*) or *two* (**) *asterisks* represent the *P* values of *T* test for less than 0.05 or 0.01, respectively

Development of InDel/SNP markers and phylogenetic analysis

The linkage markers for the QTLs detected in the DX-BIL population, plus the InDel and SNP genome-wide markers were employed to genotype the DO-RIL population, Dongye1 and its parents, as well as other common wild rice accessions and representative cultivated varieties. The InDel and SNP markers were developed according to the description by Mao et al. (2011), and are listed in Supplement Spreadsheet 3.

Using the marker genotypes, a phylogenetic tree of wild rice species and representative cultivated varieties was constructed using the neighbor-joining method implemented in ClustalW (Larkin et al. 2007) and displayed using MEGA (Tamura et al. 2007). The genetic distances between wild and cultivated rice accessions were calculated with the software TASSEL 5.0 (Bradbury et al. 2007). According to the clustering algorithms proposed by Edgar (2010), the average of the wild accession to each *temperate japonica* (*Tej*) cultivar was referred to as the genetic distance between the wild rice accession and the *Tej* subpopulation. The Pearson correlation analysis and Student's *t* test were performed with Microsoft Excel.

Results

The performance of DXWR and its derivative cultivar DY1

He et al. (1996) and Chen et al. (2002) have demonstrated that DXWR possesses strong cold tolerance based on the seedling survival rate after cold stress or survival after overwintering. In our study, after 6 days duration of cold stress, DXWR seedlings had a 100 % survival rate without any chlorosis, while seedlings of the cultivated rice line XQZB were almost completely withered, showing the strong cold tolerance of DXWR at the seedling stage (Fig. 1a; Table S1). DY1, the line derived from DXWR, also possessed strong cold tolerance. Following 6 days of cold stress, both DY1 and its *Japonica* parent 0298 still had more than 90 % survival rate, in contrast to lines XQZB and 1504, in which few seedlings survived. When the cold stress was extended to 10, 12, and 16 days, DY1 was significantly more tolerant than its parent 0298 ($P < 0.05$). There were no statistically significant differences in cold stress tolerance between 1504 and XQZB (Fig. 1b; Table S1).

The seedling cold tolerance of lines in the genetic populations was of irregular distribution, showing neither a binomial nor a normal distribution (Fig. S3). That may be because hard cold stress resulted in the death of seedlings. Pearson correlation analysis showed that the phenotypes resulting from three degrees of cold stress were significantly correlated to one another based on CRI or SUR, and the CRI was extremely significant relevant to SUR within the same degree of cold stress ($P < 0.01$). Moreover, the correlations for CRI among the different degrees of cold stress were higher than those for SUR (Table S2). Thus, compared to SUR, CRI was a better criterion to score for cold resistance.

Development of SLAF markers and construction of a high-density genetic map

From the high-throughput sequencing data for the SLAF markers, the sequencing depth of the parents was ~67.3-fold and that of the offspring was ~4.0-fold. A total of 4.7 GB of data containing 46,594,650 paired-end reads was obtained, with each read being ~55 bp in length. After deleting the poor

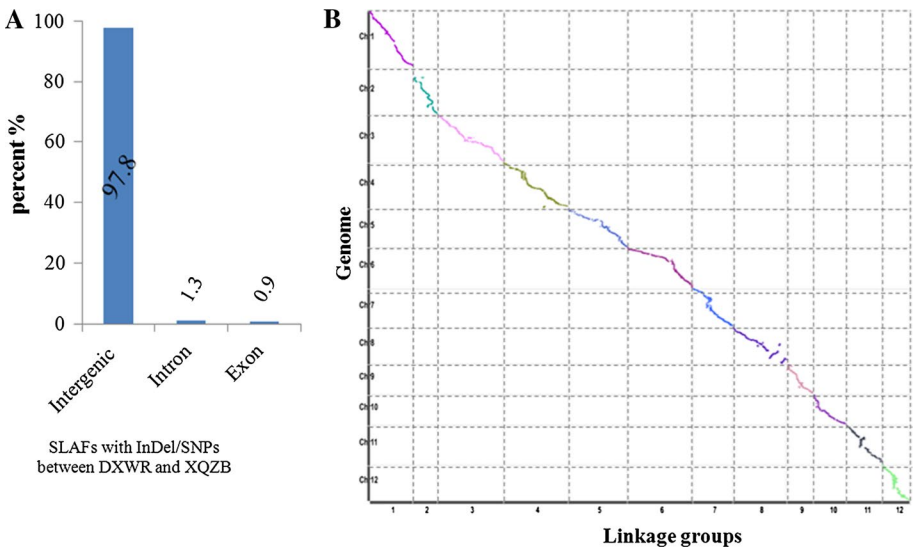

Fig. 2 SLAF markers used in the linkage map construction. **a** The percentage of SLAFs showing polymorphisms between DXWR and XQZB. **b** Colinearity between the rice genome and the linkage map derived from the DX-BIL population

quality data, a total of 50,109 SLAF markers were developed, and 21.0 % (10,519) of the markers were polymorphic between the two parents. Of these polymorphic markers, 97.8 % located to the intergenic regions, while 1.3 and 0.9 % were located in intron or exon regions of genes (Fig. 2a). Ultimately, a total of 4740 SLAFs, which were evenly distributed in the rice genome, were selected to construct a genetic map in the DX-BIL population. All of the polymorphic SLAFs are listed in the Supplement Spreadsheet 4.

The genetic map derived from the BIL population consisted of 12 linkage groups corresponding to the 12 chromosomes of rice. The total map length was 1537.1 cM, and the shortest and longest linkage groups in terms of genetic distance were chromosomes 2 (69.4 cM) and 3 (188.4 cM), respectively. The average distance between adjacent markers was 0.32 cM and the regions with distances >5 cM were less than 0.2 % of the map (Table S3). Additionally, the colinearity between the rice reference genome (*Japonica* rice cultivar Nipponbare) and the genetic map for this population was performed using the sequences of the SLAF markers in the genetic map by the Short Oligonucleotide Alignment Program (SOAP, Li et al. 2008). The results showed that the marker arrangement and orders were consistent between the maps and genomes except the chromosomal variations on chromosomes 8 and 11, and also that the SLAF marker loci covered most of the rice genome except for a region of ~7 Mb in length on chromosome 2, where no SLAF marker was located (Fig. 2b). However, InDel marker PCR assays showed that both DXWR and XQZB gave unique amplification products, suggesting that there was no deletion of a large region from the genomes of DXWR or XQZB (Fig. S4). The cause of this phenomenon is still open to interpretation. Not to miss any QTLs in this 7-Mb region, single marker analysis was performed using the InDel markers in the DO-RIL populations (Table 2).

QTLs for cold resistance in the DX-BIL population

From interval mapping in the high-density map, a total of 15 QTLs were mapped onto chromosomes 2, 3, 7, 8, 9, 11, and 12 in the DX-BIL population. Of these QTLs, 11 could be detected for both CRI and SUR, while two QTLs were detected only for each of CRI or SUR (Figs. 3, S5). There was one QTL mapped on chromosomes 8 and 9, two QTLs each on chromosomes 2 and 11, and three each were mapped onto chromosomes 3, 7, and 12. Of these QTLs, the additive effects ranged from 0.21 to 0.61 for CRI, 9.5–26.9 % for SUR, and they explained 13.8–35.7 % of the variation (Table 1). When the duration of cold stress changed, the number of QTLs detected varied accordingly. Under cold treatment at 8 °C for 4, 6, or 8 days, a total of 8, 10, and 3 QTLs were identified, respectively, suggesting that the number of QTLs detected is dependent on the duration of cold stress, and that a moderate degree is better for QTL mapping.

Single marker analysis by the candidate QTL-linked markers in the DO-RIL population

To determine whether the QTLs were also present in another DXWR-derived population, InDel markers were designed from the position of the QTL intervals and used for SMA in the DO-RIL population that consisted of 146 lines. As shown in the Table 2, except for *qCTS7.3* and *qCTS9*, the other QTLs identified in the DX-BILs were readily detected in the DO-RIL population. Under cold stress at 8 °C for 4, 6, or 8 days, a total of 10, 11, and 8 QTLs were detected, respectively, in the DO-RIL population. Four QTLs, *qCTS3.1*, *qCTS8*, *qCTS11.1*, and *qCTS11.2* were detected under all durations of cold stress and were highly significant ($P < 0.01$), suggesting that they

Fig. 3 QTL mapping for seedling cold tolerance using the CRI trait

were major QTLs for cold tolerance. *qCTS7.2* was identified in all three kinds of cold stress, but was only extremely significant in the 6-day cold stress treatment. Another five QTLs, *qCTS2.1*, *qCTS2.3*, *qCTS3.3*, *qCTS12.1*, and *qCTS12.3* were detected in only two of the cold treatments, while *qCTS2.2*, *qCTS3.2*, *qCTS7.1*, and *qCTS12.2* were only found in one cold treatment and also had minor additive effects, suggesting they were minor QTLs for cold tolerance (Table 2; Supplement Spreadsheet 5). Surprisingly, *qCTS9*, a major QTL for cold tolerance in the DX-BILs, could not be detected in the DO-RIL population. This might be explained by the fact that line 1504 appeared to be somewhat ($P = 0.064$) more cold tolerant than line XQZB under 4 days of cold stress, suggesting that line 1504 may also contain *qCTS9*, with a genetic effect similar to that of *qCTS9* QTL in DXWR (Fig. 1b).

Cold resistance QTLs pyramided in the cold-tolerant variety DY1

As shown in the Fig. 1, DY1, the line derived from DXWR is more tolerant to low temperatures than is its *Japonica* parent 0298. Thus, we tried to determine how many QTLs from DXWR were pyramided in DY1. A total of 92 Indel or SNP markers, including 28 for QTL intervals and 64 for background were used to genotype DY1. According to the genotypes of these markers, approximately 37.2 and 62.8 % of loci in the DY1 came from DXWR and from 0298, respectively. Entire regions carrying *qCTS2.3*, *qCTS3.1*, and *qCTS8*, and partial regions for *qCTS11.1*, *qCTS11.2*, and *qCTS12.2* were derived from DXWR, while the regions containing other QTLs in the DY1 genome were from 0298 (Fig. 4). This pyramiding of QTLs may explain why DY1 is more tolerant to low temperature than its cultivated parent 0298. It also suggests that these QTLs for cold tolerance are uniquely present in DXWR, or that the DXWR alleles of these QTLs had stronger additive effects than those in common *Japonica* rice, which would be confirmed in the backcrossing population of DY1.

Phylogenetic analysis of the strong cold tolerance in DXWR

To understand how DXWR evolved strong cold tolerance, DXWR and other common wild rice accessions plus the control cultivated rice lines were subjected to

Table 1 Detailed information for seedling cold tolerance QTLs detected in the DX-BIL population

QTL	Chr[a]	Interval/cM		Flanking markers		8 °C*4 days				8 °C*6 days				10 °C*8 days			
						LOD	*P*/cM[b]	Add[c]	*V*/%[d]	LOD	*P*/cM	Add	*V*/%	LOD	*P*/cM	Add	*V*/%
qCTS2.1	2	13.9	14.1	MK178244	MK192291					4.1	14.0	0.47 (25.9)	18.5				
qCTS2.2	2	44.5	45.1	MK164548	MK185933					3.1	44.9	0.44	14.4				
qCTS3.1	3	15.4	19.6	MK160280	MK137366	3.5	16.9	0.57	17.6								
qCTS3.2	3	69.7	74.3	MK152400	MK132765									4.1	73.3	(12.2)	18.6
qCTS3.3	3	166.8	174.9	MK149624	MK137032	3.7	170.6	0.58 (20.2)	18.7								
qCTS7.1	7	13.4	16.7	MK89914	MK84046	5.7	14.4	0.61 (21.4)	27.0	3.4	14.4	0.31 (15.5)	15.5				
qCTS7.2	7	43.1	57.1	MK80004	MK80344	4.1	46.0	0.59 (18.8)	20.4	3.0	48.4	0.34 (21.2)	13.8				
qCTS7.3	7	82.1	94.4	MK85382	MK81257					4.2	83.6	0.45 (25.3)	18.7				
qCTS8	8	78.3	85.0	MK36963	MK25274	3.2	80.5	0.49 (16.8)	16.1								
qCTS9	9	57.0	59.3	MK199034	MK197599					3.9	58.1	0.53 (26.9)	17.3	8.5	58.1	0.41 (19.3)	35.7
qCTS11.1	11	38.4	48.7	MK288304	MK295085									4.0	42.1	0.21 (9.5)	18.5
qCTS11.2	11	57.1	67.4	MK293906	MK288911					3.2	63.7	(18.3)	14.5				
qCTS12.1	12	20.2	25.2	MK240278	MK253183	3.6	22.0	0.55 (14.7)	18.2	4.4	22.1	0.38 (16.2)	19.7				
qCTS12.2	12	32.0	36.9	MK244291	MK238924	6.5	34.3	0.53 (17.9)	30.2	3.8	34.3	0.27 (17.9)	17.0				
qCTS12.3	12	39.7	43.7	MK238050	MK251857	6.3	41.6	0.53 (17.3)	29.7	5.3	42.1	0.29 (16.9)	22.9				

[a] Chromosome

[b] QTL positions at the peak of LOD

[c] Additive effect, values inside and outside of the parentheses represent SUR or CRI additive values, respectively (only one value indicates that the QTLs were scanned for only one trait)

[d] The percent of total phenotypic variance explained by the QTLs

Table 2 Single marker analysis of cold-tolerant QTLs in the DO-RIL population

QTLs	Markers	The significance of SMA in DO-RILs					
		SUR[f]	CRI[f]	SUR[s]	CRI[s]	SUR[e]	CRI[e]
qCTS2.3	R2ID0082						
	R2ID0268						
	R2ID0371						
	R2ID0562			*	*		
	R2ID0682		*	**	***		
qCTS2.2	R2M26_1522					*	*
	R2ID1601					*	*
qCTS2.1	R2ID2735	*	**			**	**
	R2ID2754						
qCTS3.1	R3ID0332			*	*		
	R3ID0367	**	*				
	R3ID0396	**	**	***	**	**	**
qCTS3.2	R3ID1662						
	R3ID1726			*	*		
qCTS3.3	R3ID2997						
	R3ID3054	**	**			**	**
	R7ID0431						
	R7ID0530						
	R7M7_0671						
qCTS7.2	R7ID1840			*	*	*	*
	R7ID1847		*	***	**	*	*
qCTS7.1	R7ID2122						
	R7ID2633			*	*		
	R7ID2664						
qCTS8	R8ID1184	*	**	**	**	***	***
	R8ID1243	*	**	***	**	***	***
qCTS9	35010PR						
	R9ID2124						
qCTS11.2	R11ID0885	**	***	***	***	**	**
	R11ID0888	**	***	***	***	**	**
	R11ID1009	**	***	***	***	**	**
qCTS11.1	R11ID1787	*	*	**	*	**	**
	R11ID1894	*	*	**	**	***	***
	R11ID2123		*	*	**	**	**
qCTS12.1	R12ID0470				*		
	R12ID0550		*	*	**		
	R12ID0674						
qCTS12.2	R12ID1255			*	*		
qCTS12.3	R12ID1888		*		*		
	R12ID1983	*	*		*		

*, **, *** Represent the *P* values for SMA <0.05, <0.01, and <0.001, respectively

[f,s,e] SUR and CRI represent 4, 6, and 8 days of cold treatment, respectively

a phylogenetic analysis using the genome-wide markers. Using 48 markers not linked to QTLs but distributed throughout the genome, the cultivated rice lines were grouped into five subpopulations, which are *aus*, *indica*, *aromatic*, *tropical japonica* (*Trj*) and *temperature japonica* (*Tej*), as described by Garris et al. (2005). One group of the wild rice accessions clustered with either the *Indica* subgroup (including *aus* and *indica*), or the *Japonica* subgroup (including *aromatic*, *Trj* and *Tej*), while the remaining ones occupied an intermediate position (Fig. 5a). Huang et al.

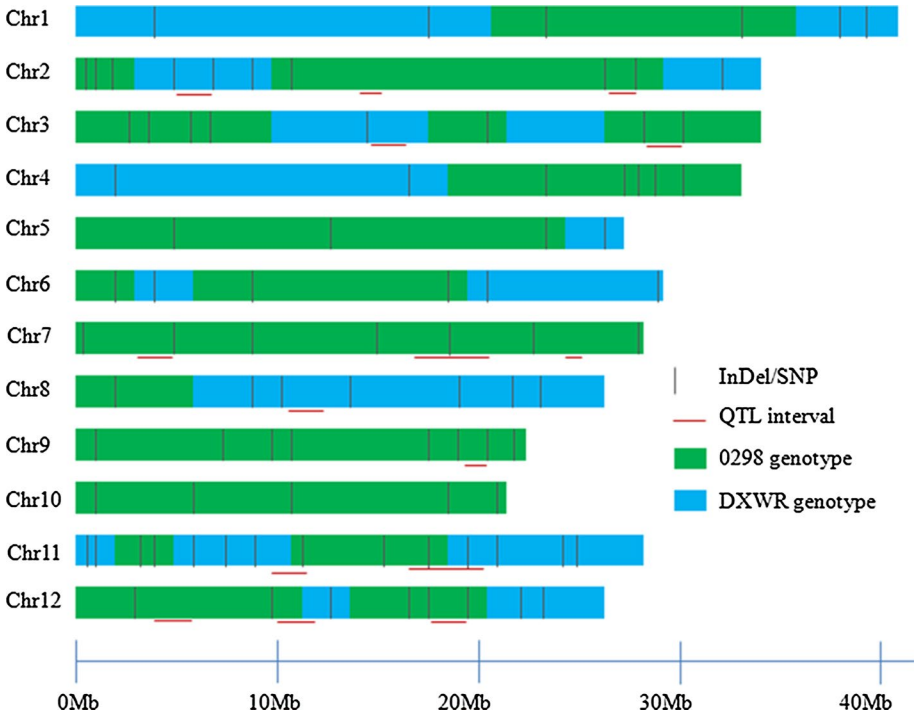

Fig. 4 Identification of the genetic basis of DY1 using background and QTL-linked markers

(2012) have stated that the progenitors of the *Indica* and *Japonica* rice subspecies cultivars are derived from the Or-I and Or-III common wild rice groups, respectively. So the common wild rice accessions that group with *Indica* rice, such as Seq225 and Seq243, belong to the Or-I ecotype, while these grouping with *Japonica* rice, such as DXWR, CLWR, and Seq191, belong to the Or-III ecotype. In other words, DXWR, CLWR and Seq191 are close to *Japonica* subspecies globally. Moreover, DXWR, CLWR, and Seq191 are much closer to the *Tej* subpopulation, which is more cold tolerant than the others (Fig. 5a; Supplemental Spreadsheet 2). When the 17 QTLs-linked markers were used for phylogenetic analysis, DXWR was maintained in the group of the Japonica rice, but moved into the center of the *Tej* subtree (Fig. 5b), suggesting that DXWR is much closer to *Tej* subpopulation within the cold-tolerant QTL regions, and its QTLs for cold tolerance originated from the ancestor of *Japonica* rice during natural selection.

As Nipponbare and other six accessions were clustered into the *Tej* subpopulation, we then calculated their average distances to each wild accession as the genetic distance between the wild rice accession and *Tej* subpopulation using the genome-wide markers. These distances ranged from 0.149 to 0.703. DXWR, CLWR, and Seq191 were the wild rice accessions that grouped closest to *Tej* (Supplemental Spreadsheet 6). As shown in Fig. 6, the latitudes where the individual wild rice accessions originated were significantly related to their distance from *Tej*. Also, the latitudes were significantly associated with cold tolerance. Moreover, cold tolerance in the wild rice accessions was significantly related to their distance from *Tej*. DXWR was the accession from the highest northern latitude, with the highest cold tolerance, and was phylogenetically closest to *Tej*. All of these results indicate that being a close relative to *Tej* and the accumulation of numerous cold tolerance QTLs in its genome gave DXWR the ability to adapt to an environment with lower temperatures.

Discussion

Accumulation of numerous loci for cold tolerance, including those unique to DXWR and those commonly present in rice cultivars, confers strong cold tolerance for adaptation to a high-latitude habitat

DXWR possesses strong cold tolerance and is, therefore, an excellent resource for molecular breeding of cold-tolerant rice. However, it is unclear how DXWR evolved to adapt to the low temperatures of its northern habitat. Several previous studies have attempted to dissect the genetic basis of cold tolerance in DXWR, but as yet, there is no unified conclusion. In our study, using interval mapping with a high-density linkage map, we identified 15 QTLs for seedling cold tolerance in the DX-BIL population. Thirteen of these QTLs, plus a novel one, were also detected in the DO-RIL genetic population by single marker analysis. All DXWR alleles of these QTLs increase the cold tolerance of rice seedlings. Five QTLs, *qCTS3.1*, *qCTS7.2*, *qCTS8*, *qCTS11.1*, and *qCTS11.2* were stably expressed in both

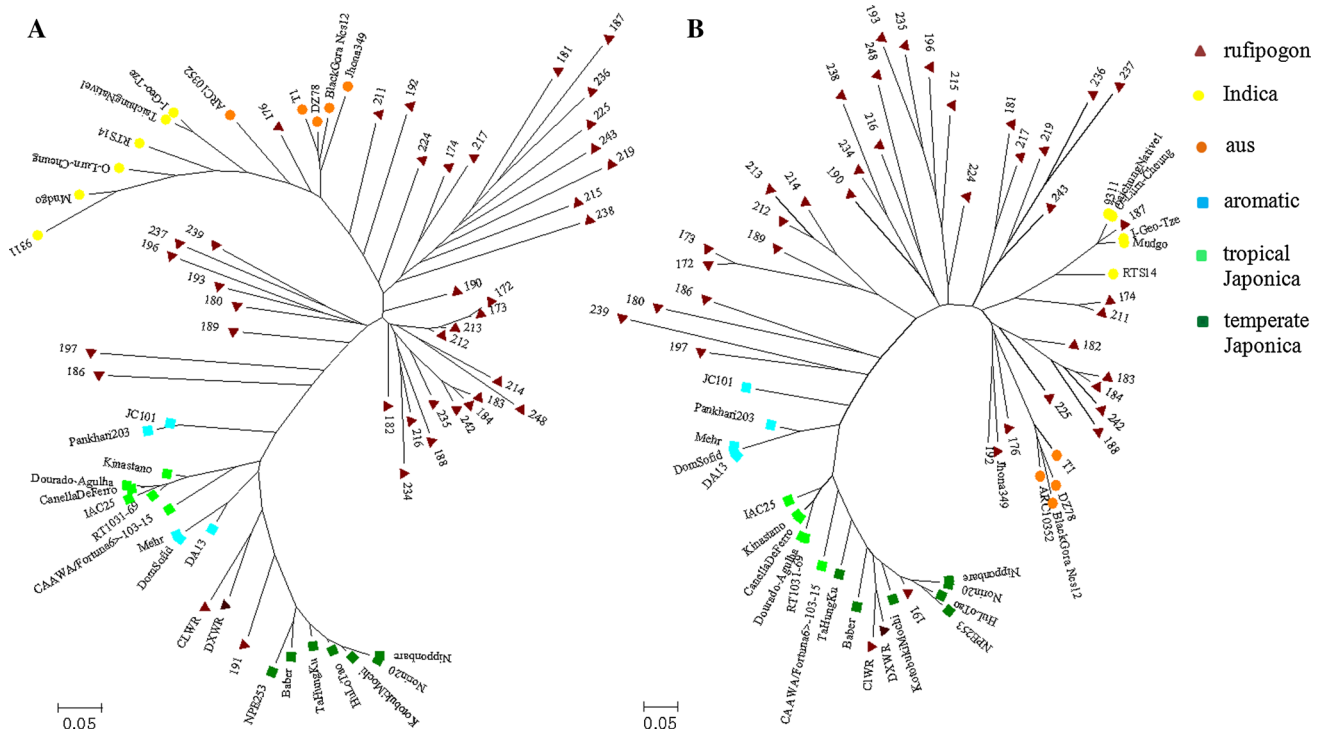

Fig. 5 Phylogenetic tree of DXWR and other wild rice accessions including control cultivated rice lines. **a** Phylogenetic tree of common wild rice and cultivated rice accessions constructed with data from background markers. *Yellow circle*, *Indica*; *Orange circle*, *Aus*; *Blue quadrate*, *Aro*; *light green quadrate*, *Trj*; *Dark green quadrate*, *Tej*; *Red triangle*, wild rice. **b** Phylogenetic tree of common wild rice and cultivated rice accessions constructed with data from QTL-linked markers. Annotation is same as above (color figure online)

genetic populations and in numerous cold treatments and can be regarded as major QTLs for seedling cold tolerance. Eight QTLs, *qCTS2.1*, *qCTS3.2*, *qCTS3.3*, *qCTS7.2*, *qCTS11.1*, *qCTS11.2*, *qCTS12.1*, and *qCTS12.2* were also detected in other *Japonica* rice lines in earlier studies (Andaya and Mackill 2003; Zhang et al. 2005; Andaya and Tai 2006; Han et al. 2007; Koseki et al. 2010; Liu et al. 2013), while another eight QTLs, *qCTS2.2*, *qCST2.3*, *qCTS3.1*, *qCTS7.1*, *qCTS8*, *qCDS7.3*, *qCTS9*, and *qCTS12.3* were unique to our study. Interestingly, all of the three QTLs in the genome of DY1 that were clearly derived from DXWR, such as *qCTS2.3* and the stably expressed QTLs *qCTS3.1* and *qCTS8* were the QTLs first detected in DXWR in our study and have not been detected in other *Japonic*/*Indica* mapping populations. These novel QTLs are likely to be the unique cold-tolerant QTLs present in DXWR, which is worthwhile to further confirm experimentally. Thus, the accumulation of these unique QTLs for cold tolerance from DXWR, and other QTLs also present in other accessions, explains the much stronger cold tolerance of DXWR compared to commonly cultivated rice lines.

Low temperature is one of the key selective pressures that determine the geographic distribution of plants in nature. There were highly significant positive relationships detected between freezing tolerance, latitude of origin of accessions, and winter habitat temperatures in Arabidopsis (Zhen and Ungerer 2008). Many cold resistance Arabidopsis genes such as *CBF2*/*FTQ4* have been shown to be QTLs for resistance to freezing, and are involved in local adaptation over a gradient of environmental temperatures (Hannah et al. 2006; Alonso-Blanco et al. 2005; Ågrena et al. 2013; Oakley et al. 2014). Each interval of *qCTS2.1* and *qCTS9*, QTLs uncovered in this study, contained one to three homologs of Arabidopsis *CBF/DREB1* genes (Mao and Chen 2012). Thus, it is worthwhile to further investigate whether these rice homologs of *CBF2* are responsible for *qCTS2.1* or *qCTS9*, and underlie the cold adaption of DXWR in high latitudes.

DXWR is likely descended from the ancestral progenitor of cultivated *Japonica* rice, and is one of the rare accessions with enough tolerance to low temperatures to survive a major short-term glacial-like condition

It has been well established that common wild rice is the progenitor of Asian cultivated rice (Khush 1997). Archeological studies support the hypothesis that the Middle and Lower Yangtze River Valley in China was the site where rice was first domesticated (Normile 1997; Zhao 1998; Lu

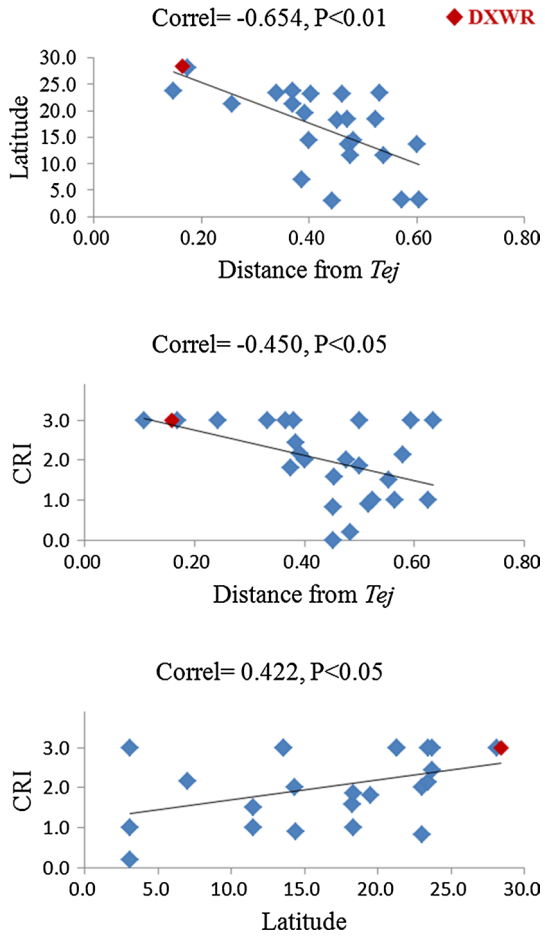

Fig. 6 Pearson correlation analysis among cold tolerance, genetic distance, and geographical locations of wild rice

et al. 2002; Jiang and Liu 2006; Zong et al. 2007). DXWR was discovered in the Middle and Lower Yangtze River Valley, suggesting that DXWR may be descended from the progenitor of cultivated rice. Temperature, especially low temperature is one of the most important limiting factors in the growth and distribution of rice. Khush (1997) proposed that cold tolerance is one of the important indicators for distinguishing between the two subspecies of rice. Our result showed that the extremely cold-tolerant wild rice, such as DXWR and CLWR, grouped with the cold-tolerant *Japonica* rice accessions, while the extremely cold-sensitive wild rice, such as Seq225 and Seq243, grouped with the cold-sensitive *Indica* lines. Moreover, the loci associated with seeding cold tolerance show that DXWR and CLWR are closer to the *temperate japonica* rice group (Fig. 5b), and the candidate genes for these QTLs may have been uniquely originated from ancestor of *Japonica* rice and have been maintained in the cold-tolerant *Japonica* rice and DXWR during natural selection. All these results suggest that the wild rice accessions that group with *Japonica* rice lines are close relatives of the *Japonica* rice progenitor, and that those grouping with *Indica* rice lines are close relatives of the *Indica* rice progenitor. Thus, the two rice subspecies may have been domesticated independently from two kinds of pre-differentiated ancestral *O. rufipogon* that differed with respect to cold tolerance. DXWR may descend from the ancestral progenitor of cultivated *Japonica* rice; this progenitor could have been an ancestral wild rice that survived a major short-term climate change during a period known as the Younger Dryas, which resulted in a return to glacial-like conditions across Northern Asia from 11,500 to 13,000 years ago (Lu et al. 2002). The colder climate would have selected for wild ancestors with the most tolerance to low temperature (such as DXWR) in the Yangtze River Valley. This hypothesis is appropriate to explain how DXWR evolved to be so tolerant to cold stress.

The QTLs identified in DXWR will be useful for molecular breeding of cold-tolerant rice

Generally, *Indica* rice is more sensitive to cold stress than *Japonica* rice. Cold resistance genes/QTLs from *Japonica* rice have been the gene resource to improve the cold resistance of *Indica* rice. However, limited genetic resources have been found for improving *Japonica* rice, in which the effects of cold stress are more severe due to its high-latitude distribution. In our study, we identified a total of 16 QTLs for seedling cold tolerance in the DX-BIL and DO-RIL populations. Among these, eight QTLs were detected in other *Japonica* rice lines in earlier studies, and thus may be common to both DXWR and *Japonica* cultivars (Andaya and Mackill 2003; Zhang et al. 2005; Andaya and Tai 2006; Han et al. 2007; Koseki et al. 2010; Liu et al. 2013). These QTLs will be useful for cold-tolerant rice breeding, especially for *Indica* rice lines. Another eight QTLs were first detected in our study and have not been detected in other *Japonic*/*Indica* mapping populations. These novel QTLs are likely to be the unique cold-tolerant QTLs present in DXWR, and will be important genetic resources for cold tolerance breeding in *Japonica* rice. This is strongly supported by the fact that six of these QTLs regions in DY1 are derived from DXWR (Fig. 4) and that DY1 is more cold tolerant than its cultivated parent 0298 (Fig. 1), though we cannot exclude that undetermined genomic segments from DXWR also play a role in cold tolerance in DY1. Cloning and characterization of target genes responsible for these QTLs will pave the way toward breeding climate-resilient rice cultivar and provide novel insights into nature adaption.

Author contribution statement C. Chen and D. Mao designed the research work, annotated the data and drafted the manuscript. D. Zhou and Y. Xiao developed the

populations. D. Mao, L. Yu, L. Li, Y. Zhu and D. Zhang performed the experiments.

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Conflict of interest The authors declare no conflict of interest.

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