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ORIGINAL PAPER

Fine mapping of the *qLOP2* and *qPSR2-1* loci associated with chilling stress tolerance of wild rice seedlings

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Abstract

Key message **Using leaf osmotic potential and plant survival rate as chilling-tolerant trait indices, we identified two major quantitative trait loci *qLOP2* and *qPSR2-1* (39.3-kb region) and *Os02g0677300* as the cold-inducible gene for these loci.**

Abstract Chilling stress tolerance (CST) at the seedling stage is an important trait affecting rice production in temperate climate and high-altitude areas. To identify quantitative trait loci (QTLs) associated with CST, a mapping population consisting of 151 BC_2F_1 plants was constructed by using chilling-tolerant Dongxiang wild rice (*Oryza rufipogon* Griff.) as a donor parent and chilling-sensitive *indica* as a recurrent parent. With leaf osmotic potential (LOP) and plant survival rate (PSR) as chilling-tolerant trait indexes, two major QTLs, *qLOP2* (LOD = 3.8) and *qPSR2-1* (LOD = 3.3), were detected on the long arm of chromosome 2 by composite interval mapping method in QTL Cartographer software, which explained 10.1 and 12.3 % of the phenotypic variation, respectively. In R/QTL analyzed result, their major effects were also confirmed. Using molecular marker RM318 and RM106, *qLOP2* and *qPSR2-1* have been introgressed into chilling-sensitive varieties (93-11 and Yuefeng) by marker-assisted selection procedure (MAS), which resulted in 16 BC_5F_3 BILs that chilling tolerance have significantly enhanced compare with wild-type parents ($P < 0.01$). Therefore, two large segregating populations of 11,326 BC_4F_2 and 8,642 BC_4F_3 were developed to fine mapping of *qLOP2* and *qPSR2-1*. Lastly, they were dissected to a 39.3-kb candidate region between marker RM221 and RS8. Expression and sequence analysis results indicated that *Os02g0677300* was a cold-inducible gene for these loci. Our study provides novel alleles for improving rice CST by MAS and contributes to the understanding of its molecular mechanisms.

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Abbreviations

CST	Chilling stress tolerance
QTL	Quantitative trait locus
PSR	Plant survival rate
LOP	Leaf osmotic potential
BIL	Backcross introgression lines
CBF	Calmodulin-binding transcription activator
DREB	Dehydration-responsive element binding

Introduction

Cultivated rice (*Oryza sativa* L.) is one of the most important crops in the world and provides 21 % of the global energy

per capita (Maclean et al. 2002). Rice is sensitive to low temperatures and is damaged when exposed to temperature below 13 °C (Caton et al. 1998; Sipaseuth et al. 2007), which leads to poor plant establishment, decreased ability to compete against weeds, delayed crop maturation, and reduced yields (Andaya and Mackill 2003a, b; Saito et al. 2004, 2010; Yoshida et al. 1996). Therefore, improving chilling stress tolerance (CST) at the seedling stage is essential for achieving yield stability and incremental advances in crop productivity.

Recently, genetic strategies have been applied to identify quantitative trait loci (QTLs) underlying low temperature in cultivated rice varieties. After exposure to cold (10 °C) for 13 days, a major QTL *qSCT-11* (logarithm of odds, LOD = 19) was identified on rice chromosome 11, which explained up to 30 % phenotypic variation of plant survival rate (PSR) (Zhang et al. 2005). Lou et al. (2007) identified five main QTLs related to cold tolerance with LOD > 4.0 on chromosomes 1, 2, and 8 under 6/10 °C for constant day/night 7 days treatment. The accumulated contribution of the five QTLs was 62.3 %. A major QTL (LOD = 15.1) was identified on chromosome 2 flanked by RM561 and RM341, which is responsible for 27.4 % of the total phenotypic variation. With the use of 191 recombinant inbred lines and 9 °C cold treatment, Andaya and Mackill (2003b) found that *qCTS12* on chromosome 12 contributes to tolerance against wilting and necrosis, whereas *qCTS4* (on chromosome 4) prevents yellowing and stunting. These two QTLs were further fine-mapped to 55 and 128-kb regions, respectively, in which eight candidate genes were identified (Andaya and Tai 2006, 2007). Over-expression of the zeta class of glutathione *S*-transferase genes in this candidate region enhanced germination and seedling growth at low temperature (Takesawa et al. 2002), suggesting that the zeta class could be the candidate genes for *qCTS12* (Andaya and Tai 2006). The above-mentioned studies suggest that rice cold or chilling tolerance is controlled by multiple genes.

A serial of QTL mapping researches relating to CST also demonstrated that the calmodulin-binding transcription activator (*CBF*) and dehydration-responsive element-binding protein (*DREB*) play important roles on plants' chilling tolerance (Alm et al. 2011; Francia et al. 2007; Knox et al. 2008; Miller et al. 2006). In *Arabidopsis*, although the *CBF/DREB1* subgroup consists of six members, only *CBF1/DREB1B*, *CBF2/DREB1C,* and *CBF3/DREB1A* are rapidly induced in response to chilling stress (Agarwal et al. 2010; Novillo et al. 2004; Shigyo and Ito 2004). Transgenic *Arabidopsis* plants constitutively over-expressing any of the three *CBF/DREB1* genes significantly improved tolerance to freezing, drought, and high salinity (Nakano et al. 2006; Shigyo and Ito 2004; Yamaguchi-Shinozaki and Shinozaki 1994). Ten putative rice *CBF* homologs (*OsDREB1A* through *OsDREB1 J*) have been identified (Mao and Chen 2012), but the functions of *CBF/DREB1* s on response to chilling stress are different even they share similar protein structure domains (Novillo et al. 2004, 2007). So far, the specific *CBF/DREB1* genes for chilling tolerance at rice seedling stage are not determined.

Wild rice (*Oryza rufipogon* Griff.) is the ancestor of cultivated rice and can be used as a donor of novel alleles for rice breeding (Nakagahra et al. 1997; Tian et al. 2006). QTL analyses have been performed to identify novel loci and genes from wild rice, and these loci are expected to be useful in improving the agronomic traits of rice, such as disease resistance, abiotic stress, and crop yield (Ashikari and Matsuoka 2006; Brar and Khush 1997; Nguyen et al. 2003). However, CST with wild rice allele introgressed into cultivated rice to elevate chilling tolerance has rarely been reported. Chilling-tolerant Dongxiang wild rice (*O. rufipogon* Griff., Dongxiang) possesses an extremely high innate tolerance to chilling stress (Li et al. 2010), and its seedlings could survive at 2 °C for 72 h (Dai et al. 2007). Therefore, we utilized Dongxiang as a chilling-tolerant donor to fine-map CST. Additionally, a new type of *CBF/DREB1G* transcription factor was identified as a candidate gene for two QTLs, *qLOP2* and *qPSR2-1*. Our results provide novel alleles for improving rice CST by MAS as well knowledge on understanding molecular mechanisms for chilling tolerance.

Materials and methods

Plant materials

In the summer of 2006, a chilling-tolerant donor parent (Dongxiang) was crossed with a chilling-sensitive variety (Nanjing 11) (Fig. 1). An advanced backcross strategy was then used to construct a primary mapping population as referenced in Robin et al. (2003) and Xiao et al. (2014). The F_1 progeny was backcrossed with Nanjing 11, resulting in 151 BC_1F_1 plants, which were grown in a greenhouse and individually backcrossed to the recurrent parent, up to BC_2F_1. During the construct period, no selection for leaf osmotic potential (LOP) and PSR was performed. A plant was selected randomly from each BC_2F_1 line, which leaded to a primary mapping population containing 151 BC_2F_1 plants. BC_2F_2 seeds were harvested individually from each BC_2F_1 plant to use for the phenotypic characterization. All plants were grown in the Yangzhou Wantou experimental fields at the Lixiahe Agricultural Research Institute of Jiangsu Province (119°42′E, 32°39′N) and Sanya of Hainan Province (110°02′E, 18°48′N).

Evaluation of leaf osmotic potential and plant survival rate

Approximately 100 BC_2F_2 seeds from each BC_2F_1 plant were equally divided into two groups (C1 and C2). To

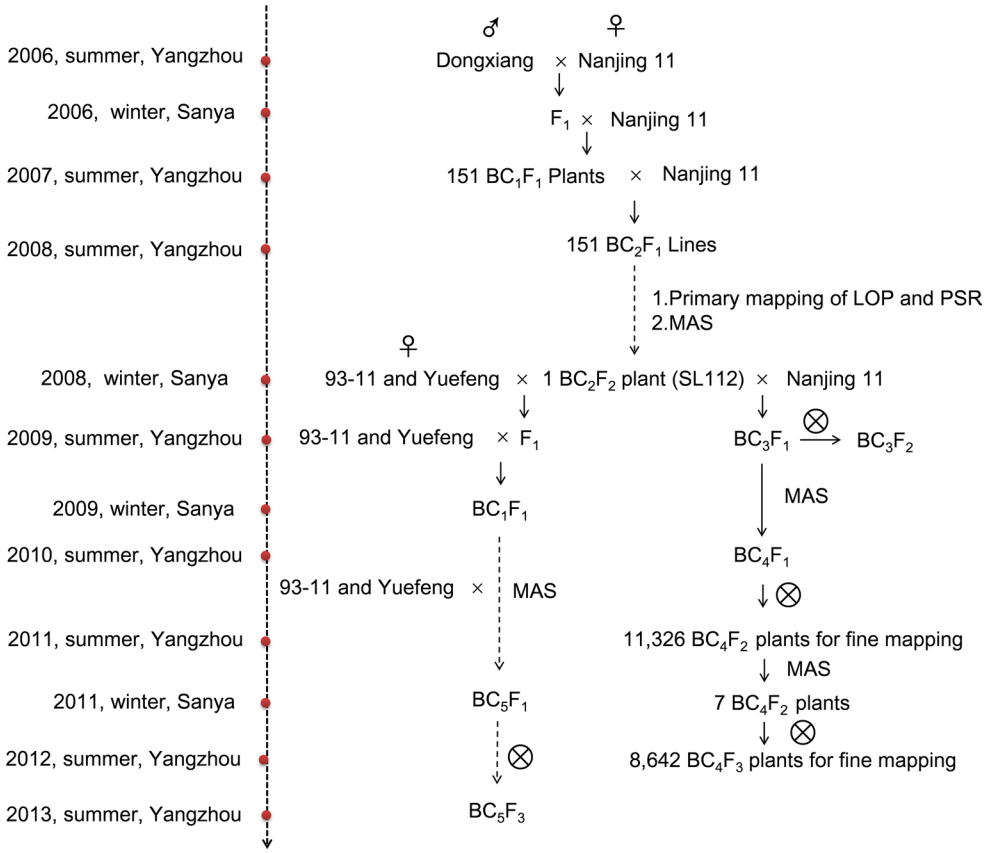

Fig. 1 Construction of the mapping population. *MAS* marker-assisted selection, *BIL* backcross introgression line (color figure online)

abolish seed dormancy, the two groups were kept at 43 °C for 1 week, then sterilized, and soaked using distilled/deionized water at 28 °C in darkness for 2 days. When the seedlings reached to the two-leaf stage, a simulated diurnal alternating illumination treatment was given: 12 h of 25,000 Lx of illumination at 4 ± 1 °C and 12 h dark at 4 ± 1 °C with relative humidity at 75–85 %. All plants were chilling-treated in a temperature-controlled phytotron growth chamber for 2 days. Then, the group C1 was immediately taken out from the chamber for measuring LOP using a permeability manometer (VAPRO 5520, Wescor, USA) which was expressed as: LOP (mmol/kg) $= 100 - \text{Ci} \times R \times (273 + T) \times 10^{-3}$, where Ci is the measured value, R is gas constant (0.08314), and T is the ambient temperature. Then, the temperature of growth chamber was adjusted back to 28/25 °C day/night (12 h each) to start the recovery process with 72 h of illumination at 25,000 Lx. After recovery, the C2 group was used to assess PSR. The plants with completely withered leaves were classified as the sensitive group, whereas plants with normal growth were classified as the tolerant group. The PSR was expressed as a percentage (0–100 %) based on the ratio of the plants showing the tolerant phenotype. All phenotypes were measured with three technical replicates.

DNA extraction and molecular marker analysis

DNA was extracted from plant leaves following the CTAB method (Rogers and Bendich 1985). All leaves were stored at −80 °C until use. All simple sequence repeat (SSR) markers were chosen from the Gramene database (http://www.gramene.org), and the sequences of the SSR markers are shown in Supplementary Table 1. PCR was performed in 20-μL reaction mixtures containing 20 ng of template DNA, 0.15 μL of 10 mM dNTPs, 2 U *Taq* DNA polymerase, 2 μL of 10 × PCR buffer [50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.01 % gelatin], and 1.5 μL of 2 μmol/L forward and reverse primers. The cycling conditions were 5 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were subjected to electrophoresis in 6 % denaturing polyacrylamide gels (Panaud et al. 1996). The gels were then silver stained as described by Xiao et al. (2011).

Linkage mapping and QTL analysis

The genetic linkage map was constructed using the MAPMAKER/EXP version 3.0 (Lander et al. 1987). QTL analysis was conducted using the composite interval

mapping (CIM) method in the QTL Cartographer version 2.5 (Lander and Botstein 1989; Lincoln et al. 1992; Wang et al. 2007) and the multiple imputation method in the R/qtl software (Sen and Churchill 2001). Significance threshold values of LOD for QTL detection were determined by using permutation tests with 1,000 replicates (Churchill and Doerge 1994). In this study, the QTL threshold of LOP and PSR was 3.2 and 2.8, respectively.

Fine mapping of *qLOP2* and *qPSR2-1*

To generate a population for fine mapping of *qLOP2* and *qPSR2-1*, molecular markers (RM106, RM318, RM267, RM274, RM44, and RM223) near the mapped QTLs were used to detect the genotype of the BC_2F_2 population. A plant (SL112) was chosen from a BC_2F_2 line, that contained a homozygous Dongxiang introgression carrying the *qLOP2* and *qPSR2-1* and five additional introgressions representing approximately 19.5 % of the Nanjing 11 genome located on 3 of the 12 chromosomes (nontarget regions). For verifying the effectiveness of *qLOP2* and *qPSR2-1*, SL112 was backcross with Nanjing 11 to produce BC_3F_2 secondary mapping population with 172 plants (Fig. 1). A BC_3F_1 plant with *qLOP2* and *qPSR2-1* was selected to construct BC_4F_2 fine mapping population consisting 11,326 plants by backcross continuously with Nanjing 11. During every backcross process, markers RM106 and RM318 were used to identify and confirm the individuals containing the *qLOP2* and *qPSR2-1* loci from Dongxiang alleles. The BC_3F_3 and BC_4F_3 seeds were individually harvested from the identified BC_3F_2 and BC_4F_2 plants, respectively, and used to measure chilling stress phenotype with three technical repeats. Additional DNA markers were needed to determine the exact position of the nearest recombination locations for *qLOP2* and *qPSR2-1*. Additional SSR markers between RM106 and RM318 were chosen from the Gramene database (http://www.gramene.org/). The sequences of the *indica* cv. 93-11 and *japonica* cv. Nipponbare were downloaded from the publicly accessible rice genome database (http://rapdb.dna.affrc.go.jp/, IRGSP 1.0, and http://www.ncbi.nlm.nih.gov/) and used to develop insertion/deletion markers using Premier v.5.0 (Supplementary Table 2).

Effects of *qLOP2* and *qPSR2-1* under different backgrounds

To further verify the effect of enhancing chilling tolerance existed in the *qLOP2* and *qPSR2-1* loci, backcross progenies were generated using SL112 as a donor parent and two chilling-sensitive *indica* varieties, 93-11 and Yuefeng as recurrent parents (Fig. 1). Two markers, RM106 and RM318, flanking *qLOP2* and *qPSR2-1*, were used in MAS to develop BC_5F_3 backcross introgression lines (BILs). Seeds from these BILs were harvested to determine chilling stress phenotype.

Expression analysis of *qLOP2* and *qPSR2-1* candidate genes

The Dongxiang, SL112, and Nanjing 11 were cultivated in the growth chamber until the two-leaf stage and were then subjected to 4 °C chilling stress. Leaf tissue (100 mg) was harvested from each line at six different chilling stress time points of 0, 3, 6, 12, 24, and 48 h. Total RNA was extracted from the samples as described by Chomczynski and Sacchi (1987). Contaminated genomic DNA was removed by DNaseI treatment. Total RNA (4 μg) was used as a template for cDNA synthesis using M-MLV transcriptase (TaKaRa Biotechnology, Dalian, China) with oligo (dT) 18 primers. Expression levels were normalized with *Actin* 1. All primer sequences for real-time PCR are listed in Supplementary Table 3. Expression analysis was operated by two biological replicates with three technical replicates. The sequences similarity between Dongxiang and Nanjing 11 alleles was analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Phenotypic evaluation of chilling tolerance

After recovery, Dongxiang and SL112 showed chilling-tolerant phenotype with normal growth leaves, but most plants of Nanjing 11 presented withered leaves (Fig. 2a). Significant differences were observed for LOP and PSR between the two parents, and their frequency distributions in the BC_2F_1 progeny were continuous and skewed toward different parent (Fig. 2b, c). Chilling-tolerant Dongxiang showed 84.7 mmol/kg of LOP and 90 % of PSR, whereas Nanjing 11 values were 60.1 mmol/kg and 12.2 %, respectively.

Detection of the Dongxiang genome among BC_2F_1 plants

Using 130 SSR molecular markers, we detected the genotype of 151 BC_2F_1 plants. Figure 3a showed that Dongxiang chromosome segments introgressed into BC_2F_1 plants covered 100 % of the whole Dongxiang genome. The percentage of the Dongxiang genome (in the heterozygous state) varied from 4.8 to 38.8 %, with an average of 23.6 % (Fig. 3b).

Identification of chilling-tolerant QTLs

The above genotype data were used to construct a genetic linkage map. All the markers were mapped to the 12 chromosomes, and the total length of 12 linkage groups was

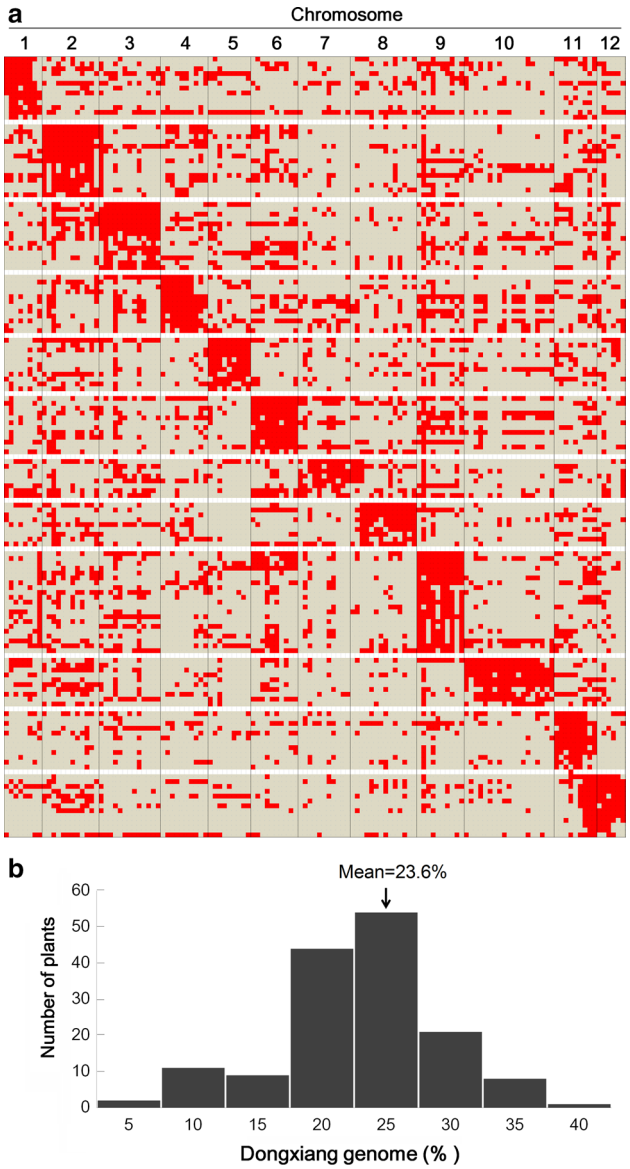

Fig. 2 Genotype of BC_2F_1 mapping population. **a** Graphical genotype of the selected BC_2F_1 plants. *Each row* represented 151 BC_2F_1 plants and *each column* represented genotype of 130 SSR markers. The *red color* indicates the heterozygous segments and the *yellow color* the homozygous regions for Nanjing 11. **b** The frequency of Dongxiang segment among BC_2F_1 plants (color figure online)

2,106.8 cM. The average genetic distance between markers was 16.2 cM, and the maximum distance between markers was 65.3 cM (RM569–RM3894) on chromosome 3 (Supplementary Table 1).

Using the BC_2F_1 phenotype data derived from the BC_2F_2 lines, five QTLs were identified by QTL Cartographer with CIM method (Table 1; Fig. 4). Among them, *qLOP2*, *qPSR2-1*, *qPSR2-2,* and *qLOP5* showed effect from Dongxiang and *qLOP8* from Nanjing 11. The percentage of phenotypic variation explained by each QTL ranged from 6.9 to 12.3 %. *qLOP8* (maximum LOD = 5.1) was located at the interval between marker RM44 and RM223 on chromosome 8 and accounted for 6.9 % of phenotypic variation. The *qLOP2* and *qPSR2-1* nearing RM106 and RM318 were identified at the same interval on chromosome 2, with contributions of 10.1 % to LOP and 12.3 % to PSR. By *R*/qtl analyzed, there were six QTLs identified on chromosome 2, 5, 8, and 9. Four QTLs were found at coincident positions of the *qLOP2*, *qLOP5*, *qLOP8*, and *qPSR2-1* regions identified by QTL Cartographer, but *qPSR5* and *qPSR9* on chromosome 5 and 9 were only checked in *R*/qtl analyzed result. It is noted that *qLOP2* (LOD = 5.0) and *qPSR2-1* (LOD = 3.9) were still identified as two major QTLs in *R*/qtl analysis, with phenotypic variation of 10.8 and 10.5 %, respectively (Table 1).

Characterization of the major effect of *qLOP2* and *qPSR2-1*

SL112 was selected to generate BC_3F_1 plant by backcrossed with Nanjing 11. After self-pollination, a small scale of BC_3F_2 secondary mapping population consisting 172 plants was developed. By CIM analysis, *qLOP2* and *qPSR2-1* were still identified as two major QTLs between RM106 and RM318, which explain 45.4 % (LOD = 21.3) and 39.2 % (LOD = 15.0) of phenotypic variation, respectively. Their major effect was also confirmed in *R*/qtl analysis result (Table 2). Therefore, *qLOP2* and *qPSR2-1* were introgressed into two chilling-sensitive cultivars, *indica* 93-11 and Yuefeng, using the marker-assisted selection procedure (MAS). This resulted in the development of 16 BC_5F_3 BILs, including nine with the 93-11 genetic background and seven with the Yuefeng genetic background. Compared with the wild-type 93-11 and Yuefeng, the values of LOP and PSR of these BILs with the *qLOP2* and *qPSR2-1* loci were significantly higher ($P < 0.01$) (Table 3), suggesting that *qLOP2* and *qPSR2-1* were reliable chilling-tolerant QTLs in different genetic backgrounds.

Fine mapping of *qLOP2* and *qPSR2-1* regions

To exclude genetic effects of *qPSR2-2*, *qLOP2,* and *qLOP5* loci from Dongxiang, a BC_3F_1 plant only with *qLOP2* and *qPSR2-1* loci was selected from the second mapping population to develop a large fine mapping group containing 11,326 BC_4F_2 plants. Markers RM106 and RM318 were used to identify recombinants around loci *qLOP2* and *qPSR2-1*, resulting in 78 recombinants having the homozygous genotype for Dongxiang or Nanjing 11 alleles. Five codominant markers (RM3793, RM3508, RM3512, RM221, and RS3) were developed between the marker RM106 and RM318 to precisely identify recombination events (Fig. 5b). Based on genotypes revealed by these markers, all recombinants were

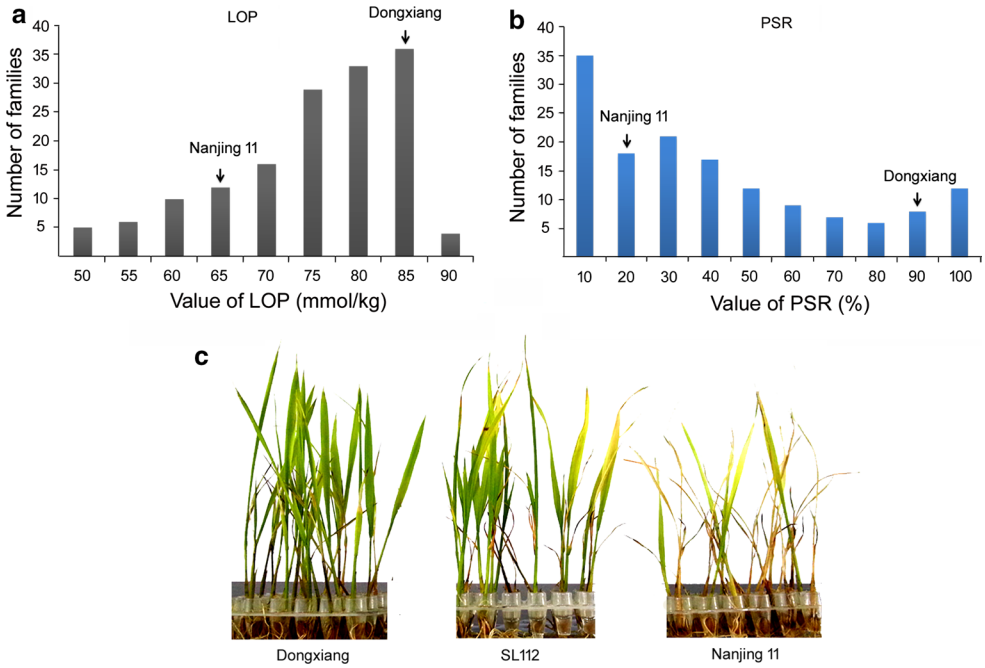

Fig. 3 The LOP and PSR frequency distribution of BC_2F_1 plants after cold treat and chilling injured phenotype of parents and SL112. **a** Frequency distribution of LOP for cold treatment in the BC_2F_2 families. **b** Frequency distribution of PSR for cold treatment in the BC_2F_2 families. **c** Phenotype of Dongxiang, Nanjing 11, and SL112 after recovery. Cold treatment conditions: 4/4 ± 1 °C constant day/night temperature (12 h) for 2 days. *Arrowheads* indicate the mean LOP and PSR of Dongxiang and Nanjing 11. *LOP* leaf osmotic potentials, *PSR* plant survival rate (color figure online)

divided into six groups (G1–6). We classified the chilling-tolerant phenotype as "T" (PSR > 60 % or LOP > 75 mmol/kg), moderately tolerant as "MT" (PSR: 30–60 % or LOP: 65–75 mmol/kg), and susceptible as "S" (PSR < 30 % or LOP < 65 mmol/kg). G1, G2, and G3 (S phenotype) showed Nanjing 11 alleles to the right of RM221, and G4, G5, and G6 (T or MT phenotype) had Dongxiang homozygous alleles to the left of RS3 (Fig. 5c). QTL analysis of these BC_4F_2 recombinants further confirmed that *qLOP2* and *qPSR2-1* located between RM221 and RS3 with the LOD of 18.6 and 25.9, which explain 63.4 and 63.9 % of phenotypic variation, respectively (Table 4).

To further delimit *qLOP2* and *qPSR2-1*, RM3512, RM221, and RS3 were used to identify BC_4F_2 plants with heterozygous for loci *qLOP2* and *qPSR2-1*. Seven plants (named G7 group) were found, and the phenotypic values varied greatly. Therefore, BC_4F_3 seeds were harvested from all G7 group plants and mixed together, which resulted in a segregation group of 8,642 BC_4F_3 plants for further fine mapping *qLOP2* and *qPSR2-1*. We developed additional codominant markers (RS8 and RS11). And RM221, RS8, RS11, and RS3 were used to screen recombinants in BC_4F_3, and seven critical recombinants were identified which fell into two genotypic classes (G7-1 and G7-2). G7-1 showed Nanjing 11 homozygous genotype on the left of RS8 and Dongxiang homozygous genotype on the right of RS11, but its phenotype is "S" (Fig. 5d). With G7-2 being "T" phenotype, showed Dongxiang homozygous genotype on the left of RS8. Using 85 BC_4F_2 and seven BC_4F_3 plants, the significant peak checked between markers RS8 and RM221. In this interval, the LOD score of *qLOP2* and *qPSR2-1* was 22.1 and 22.5 that also explained 70.0 and 67.5 % of the phenotypic variance, respectively (Table 4). So, *qLOP2* and *qPSR2-1* should be positioned to 39.3-kb region between marker RM221 and RS8 (Fig. 5d).

Identification of candidate genes for *qLOP2* and *qPSR2-1* loci

We searched for candidate genes for *qLOP2* and *qPSR2-1* using the available sequence annotation database (http://rapdb.lab.nig.ac.jp/index.html), and two genes (*Os02g0676800* and *Os02g0677300*) existed in this candidate region refer to Nipponbare genome (Fig. 5e). Real-time PCR primers were designed to evaluate the expression of each gene using Dongxiang, SL112, and Nanjing 11 under chilling conditions at 4 °C for 0, 3, 6, 12, 24, and 48 h (Fig. 6). *Os02g0676800* and *Os02g0677300* were expressed at all chilling stress period. However, only *Os02g0677300* was highly up-regulated in both parents and in SL112, and it had more rapid and dramatic response (within 6 h) to chilling stress in Dongxiang and SL112 compared with Nanjing 11. After 12 h of exposure to chilling, transcript levels began to fall in chilling-tolerant plants but remained higher than at 0 h. Hence, *Os02g0677300* may be the best candidate gene in this region. The sequencing results showed that six single-base changes occurred in the coding region, including two transversions and four transitions (Supplementary Fig. 1). Among them, only one transversion at the position 610 from the transcription start site changed the Cysteine codon TGT in Nanjing 11 to Glycine (GGT) in Dongxiang. Other mutations were synonymous (Supplementary Fig. 2).

Table 1 Chromosome location, phenotypic variances, and LOD of the QTLs for LOP and PSR at BC_2F_1 primary mapping population

QTL	Chromosome	Intervals	QTL cartographer			*R*/qtl		
			LOD	AE[a]	R^2	LOD	AE[a]	R^2
qLOP2	2	RM106–RM318	3.8	5.5	10.1	5.0	7.0	10.8
qLOP5	5	RM267–RM274	4.3	6.1	8.8	4.0	8.9	1.4
qLOP8	8	RM44–RM223	5.1	−14.0	6.9	3.2	−2.1	0.2
qPSR2-1	2	RM526–RM318	3.3	21.8	12.3	3.9	22.0	10.5
qPSR2-2	2	RM318–RM450	3.1	21.4	9.8			
qPSR5	5	RM3664–RM6082				3.16	27.11	6.4
qPSR9	9	RM11168–RM24516				3.4	12.55	3.4

AE additive effect, *LOD* logarithm of odds

[a] Negative value indicates effects from Nanjing 11, and positive values indicate effects from Dongxiang

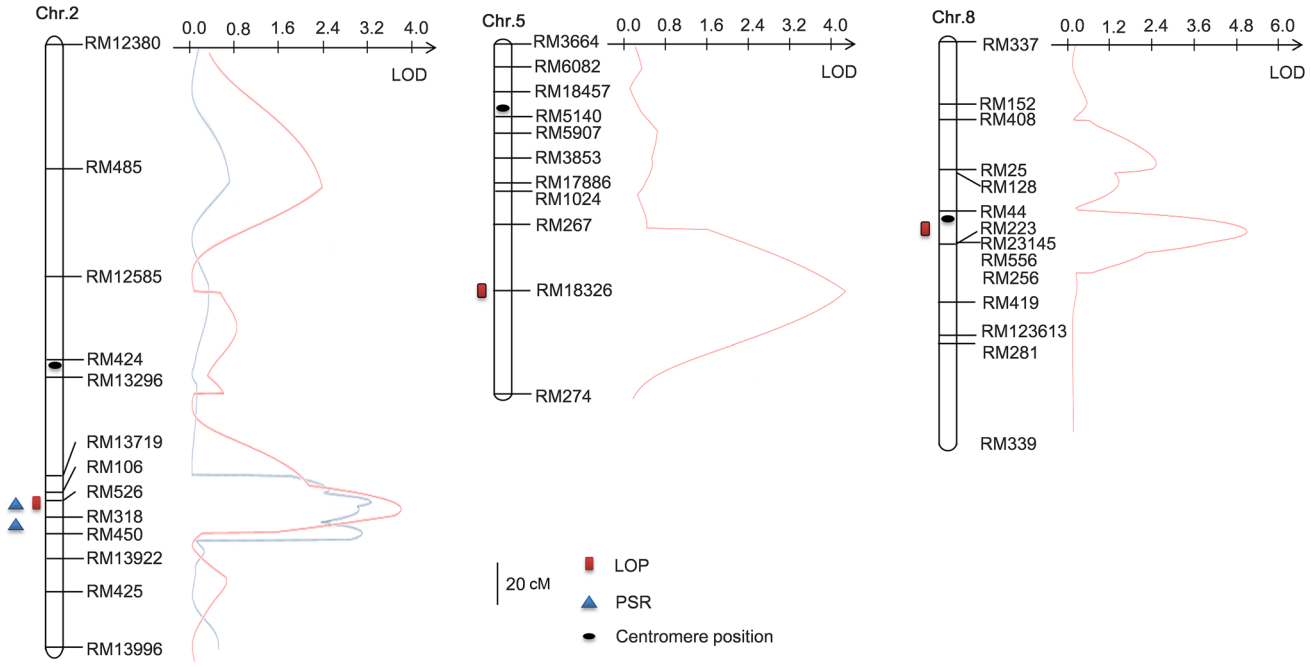

Fig. 4 Chromosomal location of putative QTLs for LOP and PSR at the seedling stage (color figure online)

Table 2 Phenotypic variances and LOD of *qLOP2* and *qPSR2-1* at BC_3F_2 second mapping group

QTL	Chromosome	Intervals	QTL cartographer			*R*/qtl		
			LOD	AE[a]	R^2	LOD	AE[a]	R^2
qLOP2	2	RM106–RM318	21.3	14.4	45.4	15.3	18.2	38.2
qPSR2-1	2	RM526–RM318	15.0	18.7	39.2	13.4	14.5	34.4

AE additive effect, *LOD* logarithm of odds

[a] Negative value indicates effects from Nanjing 11, and positive values indicate effects from Dongxiang

Discussion

Low temperature is one of the most important environment factors that affect rice productivity and its distribution. Previous genetic analyses have demonstrated that CST is controlled by multiple genes (Andaya and Tai 2007; Jiang et al. 2008; Ji et al. 2009; Koseki et al. 2010; Miura et al. 2001; Qian et al. 2000; Suh et al. 2012; Teng et al. 2001; Wang et al. 2011), but the genes that are responsible for enhancing CST have not been cloned.

A survival strategy to adopt for chilling stress involves osmotic adjustment and maintenance of cell membrane stability. Chilling-tolerant varieties can maintain membrane integrity under chilling stress (Huang et al. 2012). Reactive

Table 3 The LOPs and PSRs of BILs with 93-11 and Yuefeng genetic background

Genetic background	BIL name	LOP	PSR
93-11	BIL-1$^{93-11}$	67.2 ± 4.2*	51.9 ± 5.5**
	BIL-2$^{93-11}$	69 ± 5.5*	53.4 ± 6.8**
	BIL-3$^{93-11}$	70.1 ± 6.2*	54.6 ± 6.6**
	BIL-4$^{93-11}$	72.8 ± 5.6**	54.8 ± 8.7**
	BIL-5$^{93-11}$	73.4 ± 4.1**	54.8 ± 8.2**
	BIL-6$^{93-11}$	69.4 ± 4.3*	56.1 ± 5.5**
	BIL-7$^{93-11}$	74.6 ± 5.5**	56.3 ± 8**
	BIL-8$^{93-11}$	74.3 ± 6.2**	56.4 ± 7.3**
	BIL-9$^{93-11}$	75.2 ± 4.5**	61.8 ± 9.4**
	CK(93-11)	61.9 ± 4.8	36.8 ± 5.8
Yuefeng	BIL-1^{Yuefeng}	65.2 ± 5*	48.2 ± 6.3**
	BIL-2^{Yuefeng}	68 ± 6.6**	42.6 ± 9.4*
	BIL-3^{Yuefeng}	64.7 ± 4*	47.6 ± 5.2**
	BIL-4^{Yuefeng}	62.3 ± 5.4*	45.6 ± 7.0**
	BIL-5^{Yuefeng}	66.7 ± 1.9*	43.3 ± 10.4*
	BIL-6^{Yuefeng}	65 ± 6.4*	52.4 ± 7.9**
	BIL-7^{Yuefeng}	66.7 ± 4.5*	44 ± 6.5**
	CK (Yuefeng)	52.7 ± 3.5	22.6 ± 7.0

* $P < 0.05$; ** $P < 0.01$

oxygen species are generated during chilling stress in chilling-sensitive plants (Song et al. 2011; Theocharis et al. 2012), causing severe damage to various cellular components such as membrane lipids and structural proteins and hence irreversibly damaging the cell membrane, electrolyte leakage, and cell dehydration and death (Huang et al. 2009; Lee et al. 2004). Therefore, the degree of the plant withering after chilling stress indicates the injury to the plants (Nagamine 1991). The lower the LOP is, the more extensive is the leaf withering. Leaf curling occurred in Nanjing 11 after 6 h of treatment at 4 °C, and by 48 h, most of the leaves were totally withered. In contrast, for the chilling-tolerant Dongxiang, there was normal growth with few wilted leaves during chilling stress and the subsequent recovery stages, indicating that cell membrane integrity was better in Dongxiang and that dehydration was less. We identified three QTLs that are related to the LOP, two from Dongxiang, named *qLOP2* and *qLOP5*, and *qLOP8* from Nanjing 11. To date, no previous research has reported the use of this trait as a physiological index to map rice chilling tolerance during the seedling stage. However, this trait has been used as a physical index to map drought- and temperature-tolerant QTLs, and the mapped loci were overlapped with ours based on the physical distance. Nguyen et al. (2004), Zhang et al. (2001) identified *qOA2.1* ($R^2 = 8.9$ %, LOD = 3.0) and *qOA8.1* ($R^2 = 8.3$ %, LOD = 2.91), which are related to drought tolerance on chromosomes 2 and 8 and overlap with *qLOP2* and *qLOP8*. *qLOP5* is consistent with the QTL between marker RG182 and RG1 that is related to dehydration tolerance (Lilley et al. 1996). Therefore, the LOP can be used as a stable index for evaluating osmotic potential changes and cell membrane integrity in rice under chilling stress.

Compared with LOP, the PSR was the final phenotype reflecting the plants' adaptations to the consecutive stresses and recoveries. A correlation analysis between these parameters in BC_2F_1 plants showed a significant but not highly predictive result ($r = 0.23$, $P < 0.01$). We noted that there were two types of chilling sensitive among BC_2F_2 plants. In the first, most of the leaves withered after a chilling treatment of 48 h. In the second, there were no leaves withered during the chilling-treatment phase, but the leaves gradually withered during the restoration phase, suggesting that some plants with high LOP died during the recovery phase. The phenotypes above were reported by Koseki et al. (2010): All of the seedlings retained normal leaf color immediately after the 4 °C chilling treatment, the chilling-sensitive individuals had wilted leaves and no discernible growth after 14 days of recovery while the chilling-tolerant individuals maintained leaf color and showed growth with an increase in tiller numbers (2–3). The BC_2F_1 individuals showed LOP that was similar to that of Dongxiang but had poor survival during the recovery, which may have caused the distribution of the leaf osmotic potential and plant survival rate value to be skewed to different parents in the BC_2F_1 group. There might be different chilling-tolerant molecular and physiological mechanisms for the chilling treatment and recovery stages. After the cessation of the chilling treatment, the reversion of gene expression in the chilling-tolerant varieties was quick and easy, whereas the chilling sensitive displayed a considerably slower recovery capacity at the transcriptional level (Zhang et al. 2012). In order to preserve normal growth after chilling stress, rice should exhibit a strong ability to resist the stress during the chilling treatment and to quickly recover its metabolism during the recovery phase. Therefore, it is important to study genes that contribute to CST in both stages. In the present study, there was a significant correlation ($r = 0.76$, $P < 0.01$) between the LOP and PSR in BC_4F_2 generation, indicating that *qPSR2-1* and *qLOP2* loci play important roles in resisting chilling stress and in restoring metabolism during both the chilling treatment and the recovery stage. Han et al. (2007) found a major QTL, *qCSH2*, between RM262 and RM263 on chromosome 2 that confers seedling vigor traits under cold-water irrigation, explaining 16.6 % of the phenotypic variation. Previous studies also reported that some major chilling-tolerant QTLs located on chromosome 2. Lou et al. (2007) detected a major QTL (LOD = 15.09) flanked by RM561 and RM341 and that explained 27.42 % of the phenotypic variation for the seedling survival rate. Liu et al. (2013) also identified a major

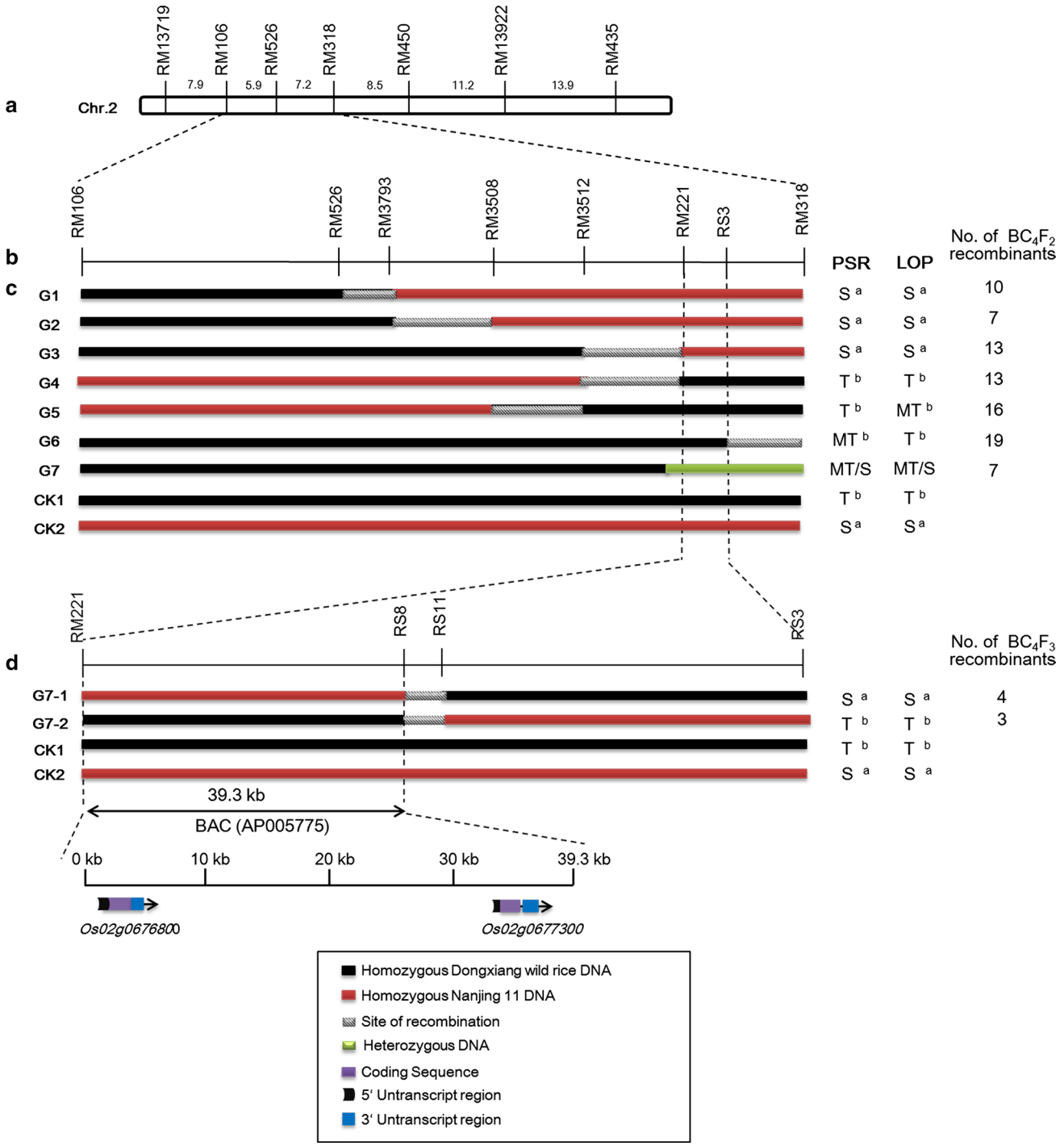

Fig. 5 Genetic and physical map covering the *qLOP2* and *qPSR2-1* loci. **a** The location of the *qLOP2* and *qPSR2-1* loci on the chromosome 2. **b** Developed polymorphic molecular markers covering this locus. **c** Progeny testing of homozygous recombinants delimited the *qLOP2* and *qPSR2-1* loci to the region between markers RM106 and RM318. The numbers of recombinants in each group and the phenotypic difference of each group from controls are shown. **d** Further fine mapping based on the group G7 and two candidate genes. CK1: Dongxiang; CK2: Nanjing 11. [a]Significant difference from CK1 with a *P* value 0.01; [b]Significant difference from CK2 with a *P* value 0.01 (color figure online)

chilling-tolerant QTL, *qCTS2* (LOD = 4.1), on chromosome 2 that accounted for 20 % of the phenotypic variation at 4 °C for 4 days. These QTLs are located on the long arm of chromosome 2, and *qCSH2* is close to *qLOP2* and *qPSR2-1*, whose physical distance was 799.4 kb according to the SSR marker sequences. These results suggest that these QTLs may be overlapped. In addition, *qLOP2* and *qPSR2-1* can significantly enhance chilling tolerance.

Table 4 Phenotypic variances and LOD of *qLOP2* and *qPSR2-1* at BC_4F_2 and BC_4F_3 mapping group

QTL	Chromosomes	Generation	Intervals	LOD	AE[a]	R^2
qLOP2	2	BC_4F_2	RM221–RS3	18.6	18.5	63.4
qLOP2	2	BC_4F_3	RM221–RS8	22.1	19.1	70.0
qPSR2-1	2	BC_4F_2	RM221–RS3	25.9	17.9	63.9
qPSR2-1	2	BC_4F_3	RM221–RS8	22.5	19.3	67.5

AE additive effect, *LOD* logarithm of odds

[a] Negative value indicates effects from Nanjing 11, and positive values indicate effects from Dongxiang

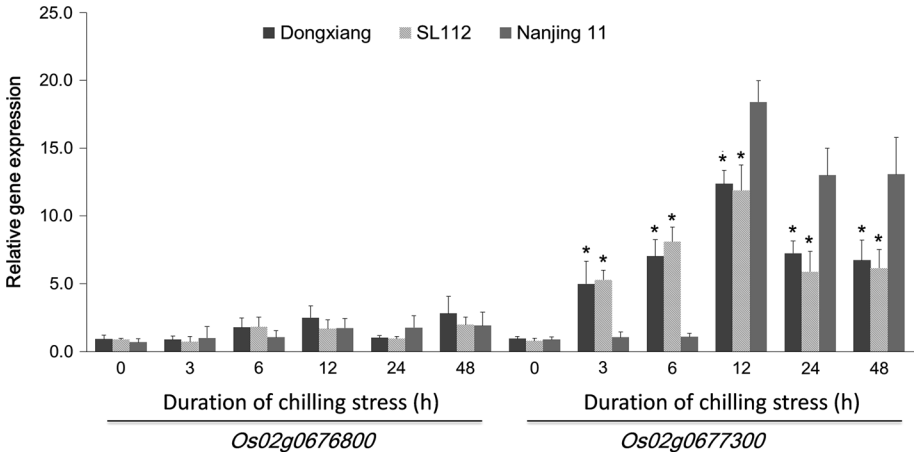

Fig. 6 Expression analysis of candidate genes by real-time PCR for plants maintained at 4 °C for different durations. *Significant difference from Nanjing 11 with a *P* value 0.01

Compare to that of the wild type, the increase in PSR ranged from 15.1 to 25 % in the 93-11 BILs containing *qLOP2* and *qPSR2-1* and from 22 to 29.8 % in the Yuefeng BILs. Therefore, further study of the function of candidate genes in *qLOP2* and *qPSR2-1* interval will confer important significance to the molecular mechanism of chilling tolerance in rice.

We identified a gene that quickly responded to the chilling stress (*Os02g0677300*) in the candidate region, and its predictive function is C-repeat (CRT)/dehydration-responsive element (DRE) binding factor1 (*CBF3*/*DREB1G*) (Mao and Chen, 2012). *CBF* belongs to chilling-induced genes that quickly responded to 0–6 h of chilling treatment and plays a crucial role in the resistance to chilling injury in rice (Chawade et al. 2013; Dubouzet et al. 2003; Wang et al. 2008; Yun et al. 2010; Zhang et al. 2009). By binding to the *CRT/DRE* regulatory elements that are located in the promoters of cold responsive (*COR*) genes, *CBF*s regulate the expression of *COR* genes in a process that involves osmolyte adjustment, detoxification of reactive oxygen species, membrane transport, and cell-protective activities (Fowler and Thomashow 2002; Fowler et al. 2005; Maruyama et al. 2004). In our study, *Os02g0667300* was up-regulated by chilling stress, in agreement with previous studies (Arvind et al. 2012). This gene responded earlier (before 12 h) in chilling-tolerant plants than in chilling-sensitive plants, indicating that Dongxiang and SL112 adapt to chilling exposure more quickly than does Nanjing 11. Zhang et al. (2012) reported highly enriched *CBF* binding motifs in up-regulated genes during early cold stress (2 h), while a decrease appeared after 12 h cold stress. Nanjing 11 lacks a rapid response to chilling stress, resulting in increased injury given that the *COR* genes were not up-regulated in time to prevent plant damage. Morsya et al. (2005) reported that OsLti6a, OsLti6b, and P5CS could be up-regulated by *CBF/DREB1*, and the expression level was directly related to the ability to resist chilling injury. OsLti6a and OsLti6b belong to a class of low molecular weight hydrophobic proteins that are involved in maintaining the integrity of the plasma membrane throughout cold exposure (Zhang et al. 2008). P5CS is a proline biosynthetic enzyme, and *CBF* over-expression also resulted in elevated P5CS transcript levels, thereby increasing proline levels in transgenic plants (Morsya et al. 2005). Proline plays multiple roles in plant stress tolerance, e.g., as a mediator of osmotic adjustment, a stabilizer of proteins and membranes, and an inducer of osmotic stress-related genes (Verbruggen and Hermans 2008; Szabados and Savoure 2010). In this study, OsLti6a, OsLti6b, and P5CS were expressed at lower levels in Nanjing 11 compared with in Dongxiang and SL112 during the chilling stress (Supplementary Fig. 3), indicating that *Os02g0677300* may be the best candidate gene for *qLOP2 and qPSR2-1* loci. The functional analysis of candidate genes is underway, and

further work will eventually provide a detailed understanding of the molecular mechanisms of rice chilling tolerance.

Author contributions NX, W-NH, and J-MC participated in the study conception and design. YG, A-HL, and YD contributed to DNA extraction and molecular marker identification. W-NH, C-HP, Z-YD, and J-HJ contributed to data analysis. NX wrote the manuscript. Y-HL and X-XZ critically revised the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest None.

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