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RESEARCH ARTICLE

Locating QTLs controlling overwintering seedling rate in perennial glutinous rice 89-1 (*Oryza sativa* L.)

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Abstract

A new cold tolerant germplasm resource named glutinous rice 89-1 (Gr89-1, *Oryza sativa* L.) can overwinter using axillary buds, with these buds being ratooned the following year. The overwintering seedling rate (OSR) is an important factor for evaluating cold tolerance. Many quantitative trait loci (QTLs) controlling cold tolerance at different growth stages in rice have been identified, with some of these QTLs being successfully cloned. However, no QTLs conferring to the OSR trait have been located in the perennial *O. sativa* L. To identify QTLs associated with OSR and to evaluate cold tolerance. 286 F_{12} recombinant inbred lines (RILs) derived from a cross between the cold tolerant variety Gr89-1 and cold sensitive variety Shuhui527 (SH527) were used. A total of 198 polymorphic simple sequence repeat (SSR) markers that were distributed uniformly on 12 chromosomes were used to construct the linkage map. The gene ontology (GO) annotation of the major QTL was performed through the rice genome annotation project system. Three main-effect QTLs (*qOSR2, qOSR3*, and *qOSR8*) were detected and mapped on chromosomes 2, 3, and 8, respectively. These QTLs were located in the interval of RM14208 (35,160,202 base pairs (bp))–RM208 (35,520,147 bp), RM218 (8,375,236 bp)–RM232 (9,755,778 bp), and RM5891 (24,626,930 bp)–RM23608 (25,355,519 bp), and explained 19.6%, 9.3%, and 11.8% of the phenotypic variations, respectively. The *qOSR2* QTL displayed the largest effect, with a logarithm of odds score (LOD) of 5.5. A total of 47 candidate genes on the *qOSR2* locus were associated with 219 GO terms. Among these candidate genes, 11 were related to cell membrane, 7 were associated with cold stress, and 3 were involved in response to stress and biotic stimulus. *OsPIP1;3* was the only one candidate gene related to stress, biotic stimulus, cold stress, and encoding a cell membrane protein. After QTL mapping, a total of three main-effect QTLs—*qOSR2, qOSR3*, and *qOSR8*—were detected on chromosomes 2, 3, and 8, respectively. Among these, *qOSR2* explained the highest phenotypic variance. All the QTLs elite traits come from the cold resistance parent Gr89-1. *OsPIP1;3* might be a candidate gene of *qOSR2*.

Keywords Overwintering seedling rate (OSR) · QTLs · Rice · SSR markers

Introduction

Overwintering is the process by which rice survives through the winter season and germinates the following spring. Whether the axillary buds can survive overwintering is a key distinction between annual and perennial plant species. Cultivated rice is an annual plant; therefore, its axillary buds are generally unable to survive overwintering. In Zhongxian, Chongqing, a special cold tolerant germplasm resource named glutinous rice 89-1 (Gr89-1, *Oryza sativa* L.) was discovered that can overwinter using axillary buds in low temperatures at different altitudes. The following spring, the plants germinate and grow again (Zhao et al. 2007). The cold

Xiaoshu Deng and Lu Gan had made the equal contribution for the research.

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tolerance of the Gr89-1 seedling was found to reach grade one (Zhao et al. 2008).

Cold tolerance during the seedling stage in rice has been studied extensively, with results identifying many quantitative trait loci (QTLs) (Liu et al. 2018). Some researchers consider cold tolerance during the seedling stage to be a complex trait involving multiple genes, whereas others believe that it is a quantitative trait; some other researchers believe there to exist additive and interactive effects of nuclear genes as well as cytoplasmic influences (Deng et al. 2009; Rao et al. 2013). With increased molecular genetic studies being undertaken on cold tolerance during the seedling stage of rice, cold tolerance has been found to be a complex trait usually controlled by multiple genes (Deng et al. 2009; Zhao et al. 2012a, b; Xu et al. 2014). Currently, great progress has occurred in QTL mapping of cold tolerance during the seedling stage. Commonly used populations include F2/F3, back cross, double haploid, recombinant inbred lines (RILs), chromosome segment substitution lines, introgression lines, and single-segment substitution lines. Whatever population is chosen, seed germination is always considered to be a phenotypic trait to evaluate seedling cold tolerance. Tolerance of the seedlings during the one-heart and two-leaf stage has been evaluated following the transfer of young seedlings to cold conditions (Xu et al. 2014; Liu et al. 2018). However, no QTL mapping of seedling cold tolerance has been undertaken for overwintering till date.

In the present study, a molecular linkage map consisting of 198 simple sequence repeat (SSR) markers was constructed using F_{12} RILs derived from a cross between Gr89-1 (cold tolerant variety) and Shuhui527 (SH527; cold sensitive variety). Then, we analysed the genetic effects regarding overwintering seedling rate (OSR). The objective of the present study was to provide references for the identification and utilization of excellent and new cold-tolerant genes.

Materials and methods

Plant materials

Gr89-1 is an important germplasm for rice breeding, which contains many resistance genes. Meanwhile, Gr89-1 has been selected in modern molecular breeding projects as an important resource resource for favourable varieties mining (Deng et al. 2015). Therefore, we chose Gr89-1 as the cold-resistant parent for the present study. Gr89-1 can survive in a naturally cold winter environment and germinates by axillary buds the following spring, is planted only once, and grows for many years. Survival rates of overwintering axillary buds and stems have been observed at different altitudes. The overwintering rice seedlings grew neatly and the yield was equal to that during the normal season (up to 6.29 t/hm2), with the cold tolerance reaching grade one during the seedling stage (Zhao et al. 2008). The overwintering ability of Gr89-1 was likely controlled by polygenes and heritabilities showed diversity in different hybrid combinations (Zhao et al. 2008, 2012a, b). The cold sensitive parent SH527, which is an excellent restorer of hybrid rice, was bred by Sichuan Agricultural University. SH527 does not survive through the cold winter season nor germinates during the following spring, is planted once a year, and is harvested only once. In the present study, a RIL population derived from a cross between Gr89-1 and SH527 containing 286 F_{12} RILs was used to construct a molecular genetic map and identify the QTLs controlling OSR.

Evaluation of seedling cold tolerance

The field trials were located at an altitude of 260 m with a subtropical monsoon climate and four distinct seasons. Forty-day-old seedlings were transplanted in a single row that contained 15 plants per line with 20 × 30 cm spacing between plants and rows. The field management followed essentially the normal agricultural practices, with fertilizer applied (per ha) as follows: 120 kg nitrogen, 97.5 kg phosphorous, and 75 kg potassium as the basal fertilizer; 47.5 kg nitrogen at the tillering stage; and 20 kg nitrogen at the booting stage. At the maturity stage, the height of the stubble for the biparents and all F_{12} RILs remained at 15 cm after the straw was cut and were exposed to the natural environment for cold winter screening. Relative moisture in the 0–20 cm depth soil ranged from 76.88 to 82.15%, and there was no standing water on the field surface during the winter. The daily temperature was 11–33 °C (average low temperature was 17 °C) in October, 5–22 °C (average low temperature was 10 °C) in November, 5–18 °C (average monthly temperature was 8 °C) in December, − 1 to 18 °C (average low temperature was 5 °C) in January, and 3–24 °C (average low temperature was 6 °C) in February. Phenotypic data of OSR were collected from each of the 286 F_{12} RILs, and calculated using the following formula:

$$\mathrm{OSR}\,(\%) = \frac{\text{Overwintering seedlings}}{\text{Rice stems}} \times 100$$

SSR analysis

Genomic DNA was extracted from the fresh leaves of RILs and parents using a modified cetyl trimethylammonium bromide method (Rogers and Bendich 1988). All SSR primers were synthesized based on the primer sequences as published by McCouch et al. (2002) at the Shanghai Invitrogen Biotechnology Company. A total of 686 pairs of SSR markers distributed evenly across all 12 rice chromosomes were used for the genotypic analysis of the RILs.

Polymerase chain reaction (PCR) amplification (Biswas et al. 2012) was performed in a 25 μL reaction mixture containing 1 ng/μL as the template DNA. Cycle parameters were as follows: 94 °C for 5 min and 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 separated by using a 3% denaturing polyacrylamide gel electrophoresis followed by silver staining (Xu et al. 2002). SSR analysis was performed based on the method described by Suh et al. (2009).

Linkage mapping and QTL analysis

The F_{12} RILs were used to identify the QTLs that controlled overwintering germination during the seedling stage. The molecular linkage map for the entire genome was constructed using MAPMAKER3.0 (Lincoln et al. 1992). A Kosambi mapping function was used to convert recombination frequencies to map distances in centiMorgans (cM). The GROUP command was set for the logarithm of odds (LOD) to 5.0 to identify initial linkage groupings and markers that did not belong to the initial group were included in the groupings at LOD = 3.0. Chi square tests were performed to examine segregation ratios at the marker loci for deviation from the expected ratio of 1:1 and were coupled with determination of skewness.

Results

Phenotypic evaluation of cold tolerance in Gr89-1 and SH527

After recovery, the cold tolerance of Gr89-1 at the one-heart and two-leaf stage was significantly stronger than that of SH527 (Fig. 1a–c). After treatment in 6 °C for 24 h, most seedling leaves of Gr89-1 were still green, whereas all the SH527 seedling leaves had turned yellow (Fig. 1a). After 72 h and 120 h in 6 °C, the SH527 seedlings had gradually died, whereas the survival rate of Gr89-1 seedlings reached up to 90% (Fig. 1b, c). Two months after harvest, no axillary buds in SH527 had survived, the stems were dry, and the leaves and leaf sheaths dehydrated and turned yellow. Three months after harvest, no axillary buds had survived and the rice pile withered. However, in Gr89-1 the lodging resistance increased, the amount of withering leaves decreased, and the leaf sheath was always green in the lower node. The next year, the overwintering Gr89-1 seedlings mainly germinated from the base of the axillary buds and the OSR was 158.65% (Fig. 1d–g).

Analysis of OSR in RIL population

After harvest, the 286 F_{12} RILs plants were left at a height of 15 cm. As time elapsed, the temperature decreased, and the

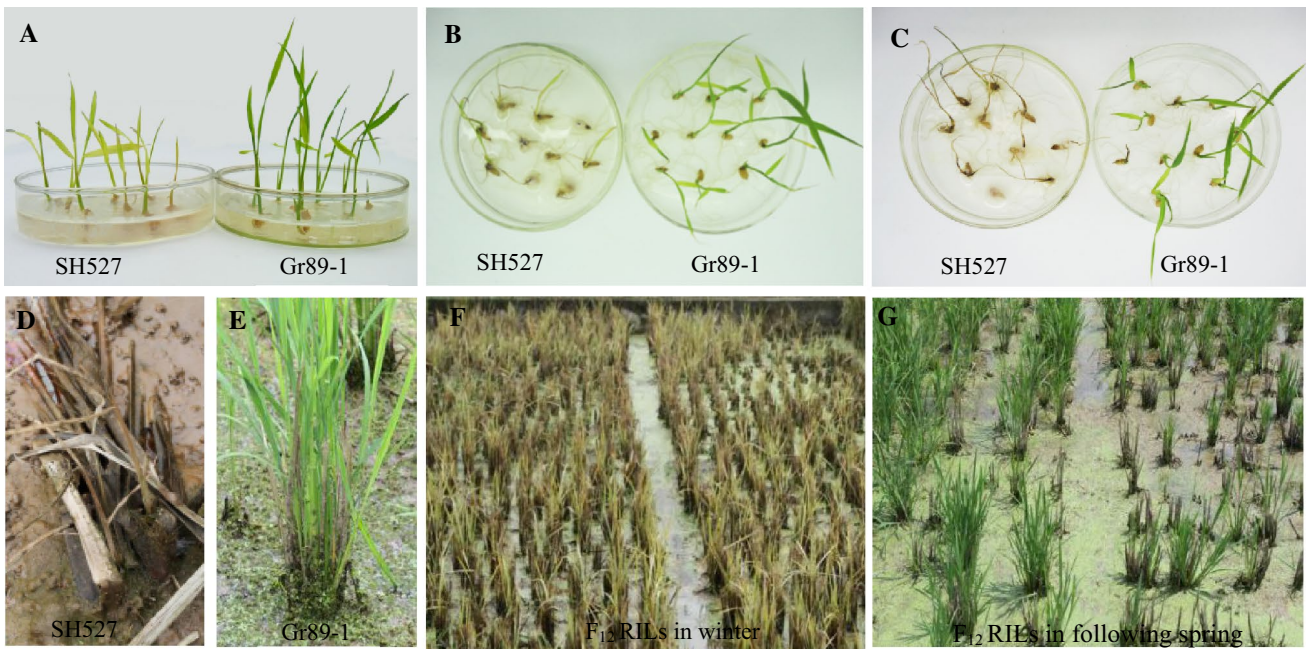

Fig. 1 Identification of cold resistance in Gr89-1, SH527, and F_{12} RILs. Phenotype of SH527 and Gr89-1 after 7 days recovery in low temperature treatment at 6 °C for 24 h (**a**), 72 h (**b**), and 120 h (**c**). Overwintering phenotype of SH527 (**d**) and Gr89-1 (**e**). Phenotype of F_{12} RILs constructed by the cross between Gr89-1 and SH527 in winter (**f**) and the following spring (**g**)

colour of the leaves and stems changed from green to yellow (Fig. 1f). Most of the axillary buds of the RILs plants survived the winter season and germinated the following spring, with only a few axillary buds dying. During the next spring, the 286 F_{12} RILs overwintering seedlings regenerated from axillary buds of the fifth and sixth uppermost internodes (Fig. 1g). The OSR of 49 of the F_{12} RILs was 0%, which accounted for 17.13% of the total (Fig. 2). The OSR of two of the F_{12} RILs had the maximum value ranging from 140 to 160%, which only accounted for 0.69% of the total. The maximum distribution frequency of OSR of populations ranged from 20 to 40%, which was approximately 69 F_{12} RILs, accounting for 24.12% of the total (Fig. 2). The variation rate of OSR ranged from 0 to 159.32%, with an average value of 41.29% (Fig. 2). The OSR showed a continuous trend towards the cold sensitive parent SH527. These results suggest that OSR is a quantitative trait that is controlled by multiple genes.

Genetic linkage map construction and QTL mapping

A total of 686 pairs of SSR markers covering the entire rice genome were used to detect the polymorphism between Gr89-1 and SH527 (Table 1). Approximately 228 pairs

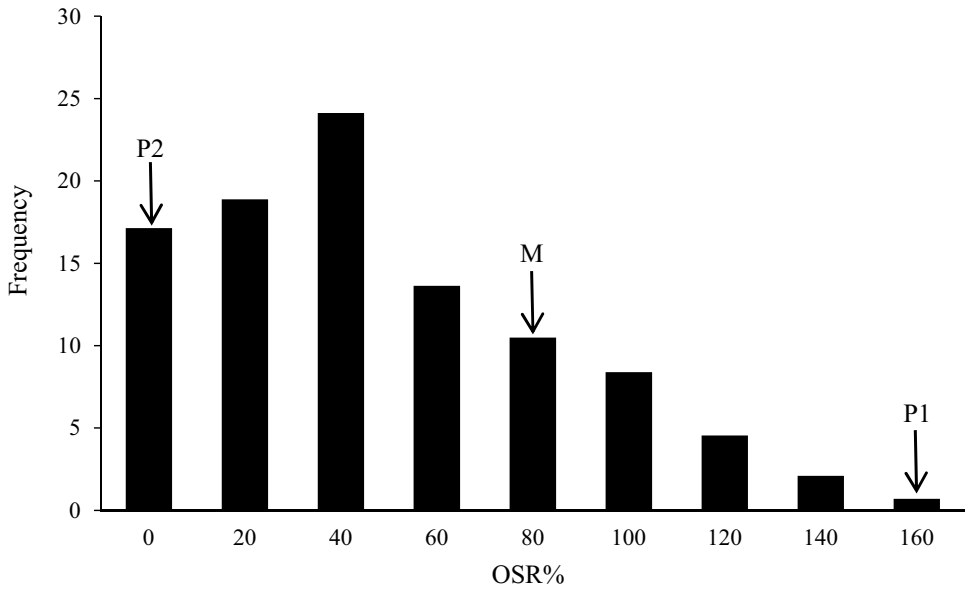

Fig. 2 Frequency distribution of OSR of the F_{12} RILs derived from Gr89-1 × SH527. Means of the RILs (M), P2 (SH527), and P1 (Gr89-1) are indicated by arrows

Table 1 Characterization of the 12 linkage groups in Gr89-1

Chr.	Map length (cM)	Marker interval (cM)			SSR markers		
		Average	Max distance	Min distance	No. of SSR primers tested	No. of polymorphisms	Polymorphic frequency (%)
1	109.2	8.40	19.2	2.6	66	13	19.70
2	180.5	5.64	25.6	1.1	78	32	41.03
3	136.1	6.48	26.6	1.1	59	21	35.59
4	151.6	10.83	28.5	3.4	53	14	26.42
5	136.8	12.44	34.5	0.6	57	11	19.30
6	141.6	10.89	45.9	1.6	43	13	30.23
7	105	11.67	39.4	2.2	39	9	23.08
8	176.4	7.35	29.8	1.6	63	24	38.10
9	119.4	5.19	13.7	1.2	66	23	34.89
10	131.5	7.31	32.5	1.6	63	18	28.57
11	103.2	11.47	20.2	4.3	50	9	18.00
12	105.5	9.59	18.2	5.5	49	11	22.45
Total	1596.8	8.06	27.8	2.3	686	198	28.86

of polymorphic markers were screened out. Then, the 198 (28.86%) pair markers that had clear polymorphic stripes and were uniformly distributed on 12 chromosomes were used to construct a genetic linkage map. The polymorphic rate of these markers varied with the chromosomes, e.g. the rate was lowest on chromosome 11 (18.00%), whereas the rate on chromosome 2 was the highest (41.03%). The genetic linkage map covered the entire rice genome by approximately 1596.8 cM with approximately 8.06 cM average interval. The longest (180.5 cM) and shortest (103.2 cM) genetic distances for single chromosomes were observed on chromosomes 2 and 11, respectively. In the marker-dense regions, the nearest markers were < 10 cM and 73.73% of the total. However, only one region showed a distance of > 40 cM between adjacent markers. All polymorphic markers were distributed on the entire rice chromosome. The genetic population and linkage map could also detect QTLs. Three QTLs for OSR (*qOSR2, qOSR3*, and *qOSR8*) were detected on chromosomes 2, 3, and 8 by using QTL IciMapping (Meng et al. 2015), which were located at RM14208 (35,160,202 bp: genomic position (bp) refers to the genome sequence of *Nippobare*)–RM208 (35,520,147 bp), RM218 (8,375,236 bp)–RM232 (9,755,778 bp), and RM5891 (24,626,930 bp)–RM23608 (25,355,519 bp), respectively (Fig. 3), and explained an average of 19.6% (LOD = 5.5), 9.3% (LOD = 2.2), and 11.8% (LOD = 3.6) of phenotypic variations, respectively (Table 2). In summary, the q*OSR2* QTL was flanked by RM14208 and RM208 on chromosome 2 with the highest LOD score, which might be a credible QTL for the OSR trait and should be given considerable attention in future studies. The additive effect of each QTL suggests that the enhanced resistance alleles were derived from Gr89-1.

Candidate genes identified in the qOSR2 interval

Based on the linked-marker information of the *qOSR2* locus, gene ontology (GO) annotation at the *qOSR2* locus was performed via the rice genome annotation project system (http://www.gramene.org). According to the database, 60 candidate genes were located at the *qOSR2* locus that was flanked by narrowing down to 359.9 kb between RM14208 and RM208 on chromosome 2 (Table 3). Among them, three genes encoded known functional proteins, and eleven were annotated as encoding expressed proteins with unknown function and other different proteins. The three known functional proteins were *OsLpa1, OsETR3* and *OsPIP1;3*, which encodes a novel protein involved in phytic acid metabolism (Kim et al. 2008), the ethylene receptor effects ethylene sensitivity (Wuriyanghan et al. 2009), and an aquaporin protein (Sakurai et al. 2005), respectively. Among the three known functional genes, *OsPIP1;3* (Sakurai et al. 2005) is associated with cold tolerance, although the mechanism for regulating gene expression is still unknown. By GO annotation, 47 functional genes involved in 219 GO terms were found and divided into three major GO categories, namely "cellular component" (58) (Fig. 4a), "molecular function" (67) (Fig. 4b), and "biological process" (94) (Fig. 4c). In the "cellular component" category, eleven candidate genes were involved in the cell membrane, which might affect the OSR (Fig. 4a; Table 4). Only three genes, *LOC_Os02g57520, LOC_Os02g57700*, and *OsPIP1;3* were involved in response to stress and biotic stimulus (Fig. 4c; Table 3). From a review of previous studies, we found seven functional genes associated with cold stress (Table 4). These are the zinc finger proteins, containing five zinc finger domains, which encode DHHC zinc finger domain containing protein, DNL zinc finger domain containing protein, RING-H2 finger protein ATL5G, ZOS2-18-C2H2 zinc finger protein, and ZOS2-19-C2H2 zinc finger protein. *LOC_Os02g57800* encode pentatricopeptide repeat containing protein, which influence the chloroplast development under cold stress (Wu et al. 2016). *OsPIP1;3* was the only candidate gene related to stress and biotic stimulus, cell membrane, and tilling stress.

Discussion

To identify QTL underlying OSR traits in perennial Gr89-1 for future gene cloning, we developed 286 F_{12} RILs derived from a cross between Gr89-1 and SH527 to map OSR and investigate the OSR phenotype. In the present study, three QTLs controlling the OSR trait were detected on chromosomes 2, 3, and 8, with *qOSR2* showing the highest LOD score. These results establish the foundation for understanding the genetic mechanism behind the OSR trait.

Regarding cold tolerance in rice, many scientists believed that it is an abiotic stress that affects the growth and development of plants and reduces yield. Low temperature impairs seed germination and reduces seedling vigour. Currently, approximately 250 QTLs controlling cold tolerance at different growth stages have been successfully identified, with some of these QTLs being successfully cloned (Biswas et al. 2017; Liu et al. 2018). There have been many studies on QTLs that control cold tolerance during the seedling stage; however, less attention has been shown to the OSR trait. A total of 98 QTLs have been successfully identified, which explained 2.7–49.3% of the phenotypic variation (Yang et al. 2015). Among these QTLs, 71 QTLs have been identified from Japonica cultivars, as these adapt to low temperatures and exhibit better cold tolerance than the Indica cultivars, and are the major rice resources for identifying genes associated with cold-tolerance during the seedling stage. However, no QTLs conferring to the OSR trait have been located in the perennial *O. sativa* L. Consequently, we first analysed the genetic basis of the OSR in Gr89-1 and found three QTLs on

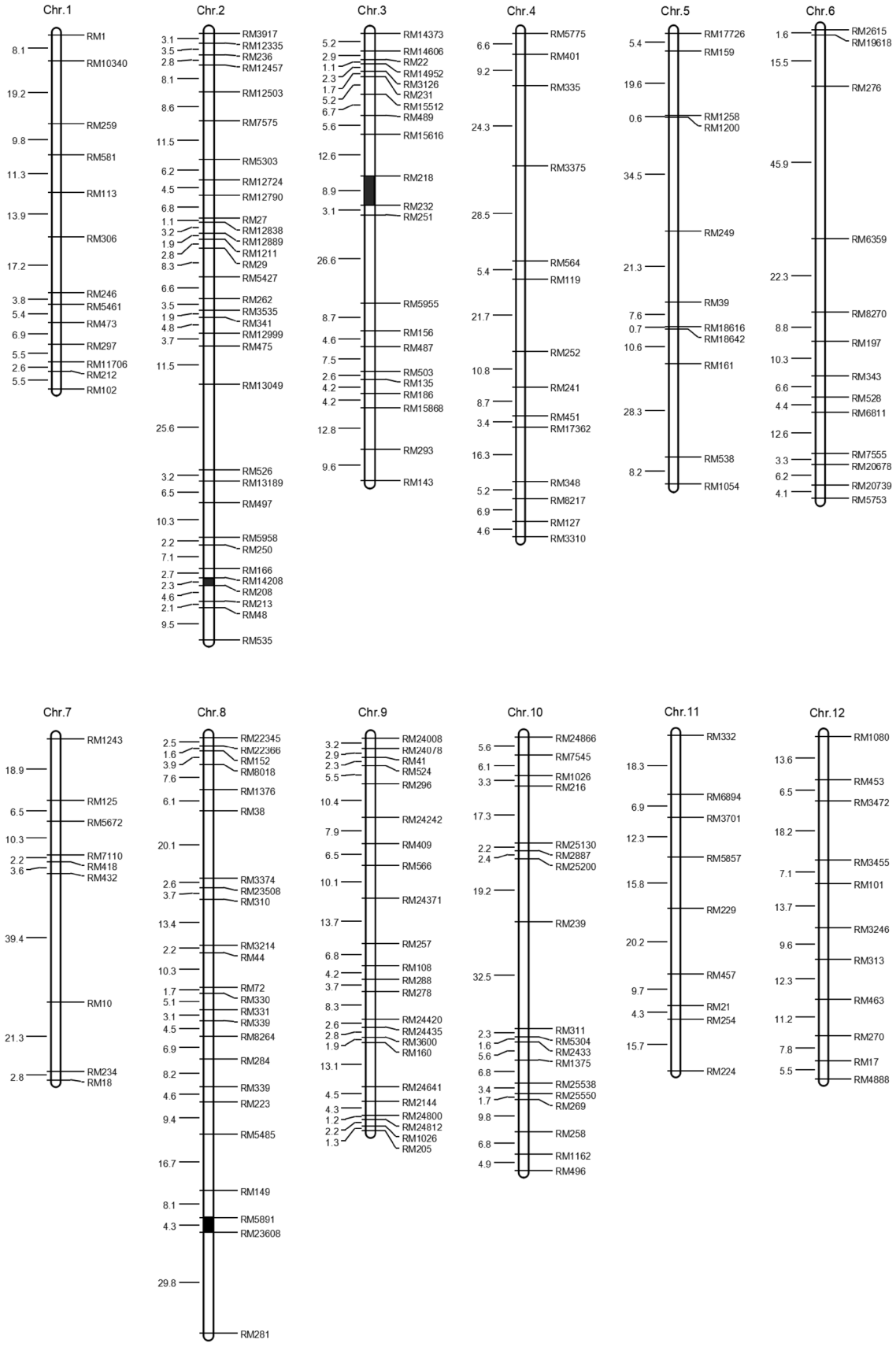

Fig. 3 Intervals distribution for QTLs of OSR

Table 2 QTLs detected for OSR after overwintering treatment by composite interval mapping

QTL	Chr.	Marker interval	Interval (cM)	LOD	PVE (%)[a]	Additive effect	Source of favorable allele
qOSR-2	2	RM14208–RM208	2.3	5.5	19.6	2.56	Gr89-1
qOSR-3	3	RM218–RM232	8.9	2.2	9.3	1.65	Gr89-1
qOSR-8	8	RM5891–RM23608	4.3	3.6	11.8	1.17	Gr89-1

[a]Percentage of the phenotypic variation explained by each QTL

three chromosomes that enriched the Gr89-1 rice seedling with cold-tolerant genes.

Cold tolerance during the seedling stage is a complex trait controlled by multiple loci and is affected not only by genetic factors, but also by the environment. Many studies have identified genes associated with cold tolerance in rice during the seedling stage, with these genes usually distributed on almost every chromosome. Qian et al. (1999) detected four QTLs related to cold tolerance during the seedling stage on chromosomes 1, 2, 3, and 4, using a double haploid population derived from a cross between *Indica* and *Japonica*. Chen et al. (2002) reported two SSR markers, RM280 and RM337, linked to cold tolerance on chromosomes 4 and 8. Han et al. (2004) detected the QTLs associated with cold tolerance in the booting stage on chromosomes 1, 2, 3, 4, 11, and 12 using a $F_{2:3}$ population derived from Minyang46/Jileng1. Hu and Wang (2005) identified five cold tolerant QTLs using RIL populations on chromosomes 1, 3, 8, and 11 with the single QTL contribution rate from 7 to 21%. A major QTL and some minor QTLs were identified by Zhang et al. (2005) and *qSCT-11* was closely linked to RM202 on chromosome 11 that explained 30% of the phenotypic variation. Andaya and Tai (2007) fine-mapped a cold tolerant QTL *qCTS4* on chromosome 4. Five QTLs were detected on chromosomes 1, 2, and 8 in a study by Qiao et al. (2007), and one of them that was flanked by RM561 and RM341 on chromosome 2 was a major locus and explained 27.42% of the phenotypic variation. Using next generation sequencing-assisted bulked-segregant analysis, Yang et al. (2013) found two QTLs on chromosomes 2 and 5. A main-effect QTL, *qSCT-3-1*, was mapped between SSR markers RM15031 and RM3400, and was detected by a chromosome segment substitution line population derived from the core collection germplasm using DP15 and DP30 as the donors and 9311 as the receptor (Zheng et al. 2011). Recently, *LOC_Os10g34840* was identified as the candidate gene for the *qPSR10* genetic locus that is associated with cold tolerance in rice seedlings (Xiao et al. 2018). These results are somewhat inconsistent, which might be due to the differences in the low temperature treatment conditions and cold tolerance evaluation criteria used in the different studies combined with various degrees of cold tolerance used to test the materials. All of these conclusions need further verification and the genetic regulation of cold tolerance in rice remains elusive.

In the present study, three QTLs (*qOSR2, qOSR3*, and *qOSR8*) on chromosomes 2, 3, and 8, respectively, were flanked by RM14208–RM208, RM218–RM232, and RM5891–RM23608 and explained 19.6%, 9.3%, and 11.8%, respectively, of the phenotypic variance. For chromosome 2, namely *qSCT-2* (Qian et al. 1999), *qCTT2* (Han et al. 2004), *qCTS-2* (Qiao et al. 2007), *qCTBP2* (Qiao et al. 2004), *LTG1* (Lu et al. 2014), *qCTS2.1/2.2* (Mao et al. 2015), and *qLOP2/qPSR2-1* (Xiao et al. 2015), controlling cold tolerance has been identified; for chromosome 3, namely *qSCT-3* (Qian et al. 1999), *qCTT3* (Han et al. 2004), *qSCT-3-1* (Zheng et al. 2011), and *qLTG-3-1* (Fujino et al. 2004); and for chromosome 8, namely *qSCT-8* (Hu and Wang 2005) and *qCTS-8* (Andaya and Mackill 2003), controlling cold tolerance has been identified. Compared with the above-mentioned QTLs, we found no OSR QTLs that were located repeatedly at the same genomic region.

In the present study, 47 functional genes were located in the region of *qOSR2*. We found 11 genes related to membrane proteins, which played an important role in maintaining the integrity of the plasma membrane under cold conditions (Ma et al. 2015; Zhang et al. 2017). The other three candidate genes were involved in response to stress and biotic stimulus. In previous studies, the zinc finger proteins especially the RING finger protein and pentatricopeptide repeat containing protein played important roles in regulating defense responses against cold stress (Mao and Chen 2012; Wang et al. 2008); therefore, the *LOC_Os02g57460* coding RING-H2 finger protein ATL5G and *LOC_Os02g57800* might also be candidate genes. The *OsPIP1;3* mRNA level increased by 60% during the cold treatment (Sakurai et al. 2005) and *OsPIP1;3* was the only one candidate gene related to stress and biotic stimulus, as well as to cell membrane and cold stress. Consequently, these candidate genes at the *qOSR2* locus might affect the OSR trait in Gr89-1, which helps us identify the main gene. In future studies, the genomic DNA sequences of the candidate genes, especially *OsPIP1;3*, can be downloaded for the designed sequencing primer. Then, the PCR product based on the biparents DNA template can be sequenced, enabling searching of the nucleotide mutation sites (A, T, G, and C)

Table 3 All candidate genes at *qOSR2* locus

Gene locus	Gene annotation	Gene locus	Gene annotation
LOC_Os02g57400	Low phytic acid 1, *OsLpa1*	LOC_Os02g57520	DNA binding protein, putative, expressed
LOC_Os02g57530	Ethylene receptor, *ETR3; Os-ETR3*	LOC_Os02g57540	60S ribosomal protein L28-1, putative, expressed
LOC_Os02g57720	Aquaporin protein; plasma membrane intrinsic protein; *OsPIP1;3*	LOC_Os02g57560	Tyrosine protein kinase domain containing protein, putative, expressed
LOC_Os02g57500	Expressed protein	LOC_Os02g57570	MATE efflux family protein, putative, expressed
LOC_Os02g57600	Expressed protein	LOC_Os02g57580	Anthocyanin permease, putative, expressed
LOC_Os02g57610	Expressed protein	LOC_Os02g57590	rRNA2-O-methyltransferase fibrillarin 2, putative, expressed
LOC_Os02g57740	Expressed protein	LOC_Os02g57620	Citrate transporter protein, putative, expressed
LOC_Os02g57780	Expressed protein	LOC_Os02g57630	Ubiquitin carboxyl-terminal hydrolase, family 1, putative, expressed
LOC_Os02g57830	Expressed protein	LOC_Os02g57640	KH domain containing protein, putative, expressed
LOC_Os02g57850	Expressed protein	LOC_Os02g57650	No apical meristem protein, putative, expressed
LOC_Os02g57870	Expressed protein	LOC_Os02g57660	Phosphatidylinositol-4-phosphate5-kinase, putative, expressed
LOC_Os02g57880	Expressed protein	LOC_Os02g57670	Ribosomal L9, putative, expressed
LOC_Os02g57900	Expressed protein	LOC_Os02g57690	Kelch repeat protein, putative, expressed
LOC_Os02g57924	Expressed protein	LOC_Os02g57700	Protein kinase, putative, expressed
LOC_Os02g57370	DHHC zinc finger domain containing protein, expressed	LOC_Os02g57710	Signal peptide peptidase-like 2B, putative, expressed
LOC_Os02g57430	DNL zinc finger domain containing protein, putative, expressed	LOC_Os02g57730	Hypothetical protein
LOC_Os02g57460	RING-H2 finger protein ATL5G, putative, expressed	LOC_Os02g57750	Protein binding protein, putative, expressed
LOC_Os02g57550	ZOS2-18-C2H2 zinc finger protein, expressed	LOC_Os02g57760	*O*-Methyltransferase, putative, expressed
LOC_Os02g57790	ZOS2-19-C2H2 zinc finger protein, expressed	LOC_Os02g57770	Glycosyl hydrolases family 16, putative, expressed
LOC_Os02g57800	PPR repeat containing protein, expressed	LOC_Os02g57810	Cytochrome P450, putative, expressed
LOC_Os02g57380	Thioredoxin, putative, expressed	LOC_Os02g57820	AT hook motif domain containing protein, expressed
LOC_Os02g57390	Pumilio-family RNA binding protein, putative, expressed	LOC_Os02g57840	Remorin C-terminal domain containing protein, putative, expressed
LOC_Os02g57410	OTU-like cysteine protease family protein, putative, expressed	LOC_Os02g57854	Vacuolar ATP synthase subunit F, putative, expressed
LOC_Os02g57420	Protein kinase APK1A, chloroplast precursor, putative, expressed	LOC_Os02g57860	OsFBX71-F-box domain containing protein, expressed
LOC_Os02g57440	LYK8, putative, expressed	LOC_Os02g57890	OsFBX72-F-box domain containing protein, expressed
LOC_Os02g57450	Ser/Thr protein phosphatase family protein, putative, expressed	LOC_Os02g57910	OsFBX73-F-box domain containing protein, expressed
LOC_Os02g57470	Tetratricopeptide repeat containing protein, putative, expressed	LOC_Os02g57930	Retrotransposon protein, putative, Ty3-gypsy subclass, expressed
LOC_Os02g57480	Transferase family protein, putative, expressed	LOC_Os02g57940	OsFBX74-F-box domain containing protein, expressed
LOC_Os02g57490	DUF260 domain containing protein, putative, expressed	LOC_Os02g57950	Hypothetical protein
LOC_Os02g57510	SNARE domain containing protein, putative, expressed	LOC_Os02g57960	Leucine rich repeat family protein, expressed

of candidate genes, and identification of the target gene functionally characterized via transgenic technology.

Cold tolerance during the seedling stage in rice is complicated and quantitatively inherited. There are obvious differences in response to low temperatures at different growth stages and in different cultivars. Therefore, it is likely that the corresponding genes are different. Results from previous studies are inconsistent because of different sources

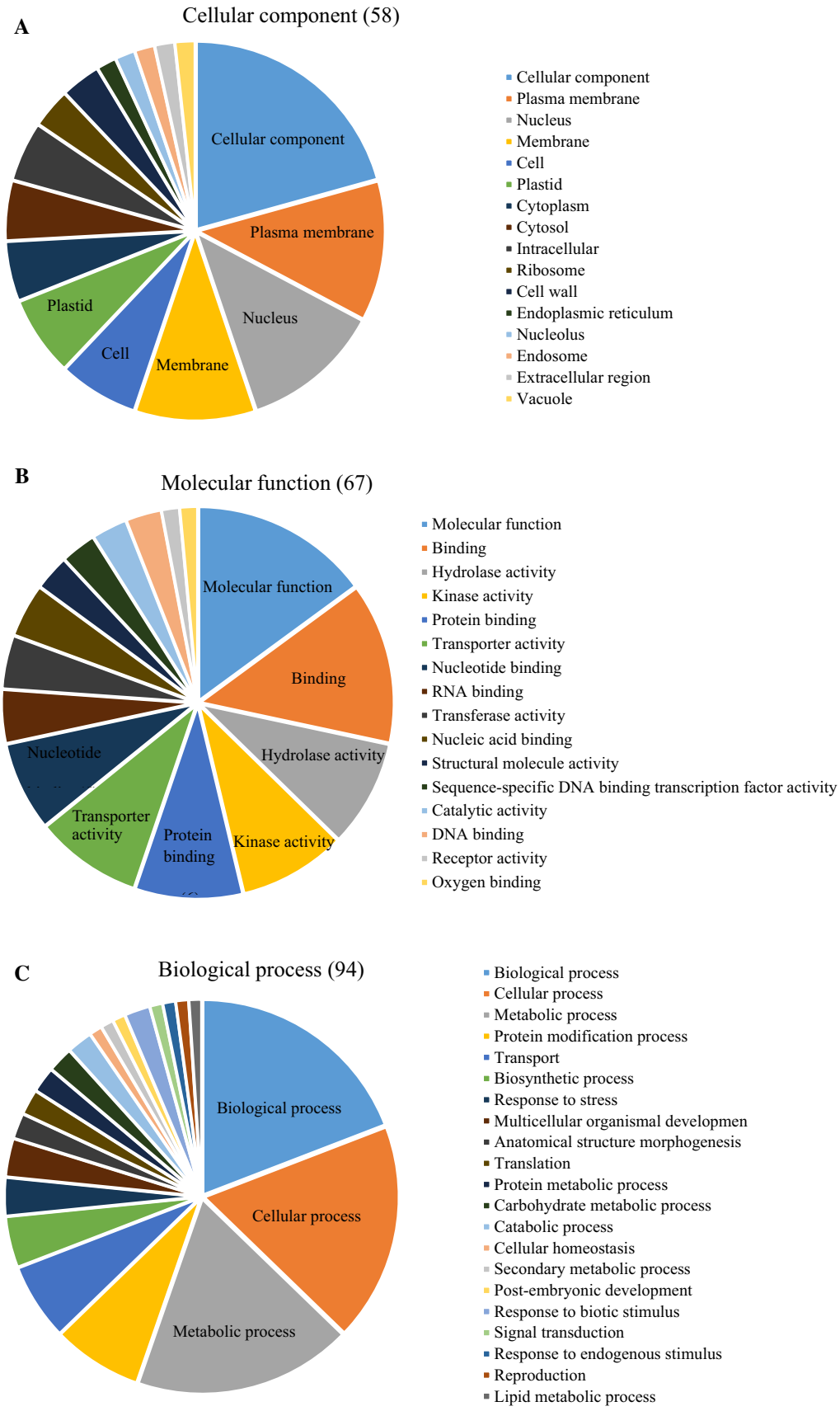

Fig. 4 Gene ontology annotation of the qOSR2. **a** Cellular component, **b** molecular function, and **c** biological process

Table 4 Candidate genes at *qOSR2* locus related to cell membrane, response to stress and biotic stimulus, chilling tolerance

Gene locus	Gene annotation	Cellular component/stress/chilling tolerance
LOC_Os02g57390	Pumilio-family RNA binding protein, putative, expressed	Plasma membrane
LOC_Os02g57420	Protein kinase APK1A, chloroplast precursor, putative, expressed	Plasma membrane
LOC_Os02g57510	SNARE domain containing protein, putative, expressed	Plasma membrane
LOC_Os02g57540	60S ribosomal protein L28-1, putative, expressed	Plasma membrane
LOC_Os02g57570	MATE efflux family protein, putative, expressed	Membrane
LOC_Os02g57580	Anthocyanin permease, putative, expressed	Membrane
LOC_Os02g57620	Citrate transporter protein, putative, expressed	Membrane
LOC_Os02g57660	Phosphatidylinositol-4-phosphate5-kinase, putative, expressed	Membrane
LOC_Os02g57710	Signal peptide peptidase-like 2B, putative, expressed	Plasma membrane
LOC_Os02g57720	Aquaporin protein; plasma membrane intrinsic protein, *OsPIP1;3*	Plasma membrane/Response to stress and biotic stimulus/Chilling tolerance
LOC_Os02g57854	Vacuolar ATP synthase subunit F, putative, expressed	plasma membrane
LOC_Os02g57520	DNA binding protein, putative, expressed	Response to stress and biotic stimulus
LOC_Os02g57700	Protein kinase, putative, expressed	Response to stress and biotic stimulus
LOC_Os02g57370	DHHC zinc finger domain containing protein, expressed	Chilling tolerance
LOC_Os02g57430	DNL zinc finger domain containing protein, putative, expressed	Chilling tolerance
LOC_Os02g57460	RING-H2 finger protein ATL5G, putative, expressed	Chilling tolerance
LOC_Os02g57550	ZOS2-18-C2H2 zinc finger protein, expressed	Chilling tolerance
LOC_Os02g57790	ZOS2-19-C2H2 zinc finger protein, expressed	Chilling tolerance
LOC_Os02g57800	PPR repeat containing protein, expressed	Chilling tolerance

of cold-tolerant genes used by the researchers. Therefore, to integrate QTLs related to cold tolerance and seek more reliable QTLs, the identification of different cold-tolerant genes is required. These results might establish the foundation for understanding the genetic mechanism behind the OSR trait. Low temperature is one of the most important environment factors affecting rice productivity and distribution. Therefore, if cold-tolerant genes in breeding are utilised and cold-tolerant varieties chosen, then rice productivity will be improved.

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