
Note

Characterization and identification of cold tolerant near-isogenic lines in rice

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To exploit the genetic mechanism of cold tolerance in rice, cold tolerant near-isogenic lines (NILs) were developed by backcrossing Kunmingxiaobaigu (KMXBG), reported to be the most cold-tolerant variety at the booting stage, as donor, with the cold sensitive Japanese commercial japonica variety, Towada. Comparisons of cold tolerance-related traits between five BC₆F₅ NILs and recurrent parent Towada under cold treatment and normal temperatures at the booting stage showed that the differences between the NILs and Towada were significant only for spikelet fertility-related traits. Analyses of cold tolerance in the NILs at the budding (germination), seedling and booting stages indicated both correlated effects and differences. Lines 1913-4 and 1916-1 showed strong and stable tolerance at all three stages. Whole genome marker screening showed that the proportion of genetic background recovery was more than 98%. Seventeen markers from KMXBG were introgressed in two or more NILs, and cold tolerance genes were possibly present in these marker regions. The NILs should be excellent materials for both rice improvement and map-based cloning of cold tolerance QTLs.

Key Words: *Oryza sativa*, cold tolerance, booting stage, near-isogenic lines.

Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. Cold stress is a common problem in rice cultivation, and is a crucial factor affecting global food production. About 30.7 million hectares of rice are grown in China and extend over a wide area ranging from 53°27'N to 18°90'N. Almost the entire area can be affected by cold injury resulting from low temperatures, and annual losses are 3–5 million tonnes of rice grain (Li and Guo 1993). Rice is a cold-sensitive plant that has its origin in tropical or subtropical areas, and cold damage can cause serious yield losses, especially when low temperatures occur during the reproductive stages. Although sowing time is usually adjusted to minimize the potential threat of low temperature damage at the reproductive stage, low temperatures still cause serious yield losses (Farrell and Lewin 2006). Critical stages for

cold injury include budding (or germination), seedling establishment, and flowering and grain filling. However, the most sensitive stage for cold injury is the booting (early reproductive) stage, especially the early pollen microspore stage, which occurs 10–12 days prior to heading. Low temperatures (15–19°C) at booting cause sterile pollen, which leads to spikelet sterility (Satake 1976).

Rice genetic resources that are tolerant to low temperatures have been identified and crossed to elite cultivars to develop cold tolerant varieties (Abe *et al.* 1989). QTL mapping studies on cold tolerance at booting have been conducted on various rice populations (Andaya and Mackill 2003, Dai *et al.* 2004, Kuroki *et al.* 2007, Liu *et al.* 2003, Mori *et al.* 2011, Saito *et al.* 1995, 2001, 2004, Suh *et al.* 2010, Takeuchi *et al.* 2001, Xu *et al.* 2008, Zhou *et al.* 2010). Although QTLs for cold tolerance at booting have been mapped on all 12 chromosomes, none has been cloned. Near-isogenic lines (NILs) are excellent materials for fine mapping and map-based cloning of the individual genetic components of complex quantitative traits. Some QTLs in rice were cloned by map-based cloning using NILs. These include *Gn1a* for grain number, *qGY2-1* for grain yield and

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qSH1 for seed shattering (Ashikari *et al.* 2005, He *et al.* 2006, Konishi *et al.* 2006, respectively).

Yunnan, located on the southwest plateau area of China, is honored as “The Kingdom of Rice” because of the high diversity of germplasm resources presumably due to isolation caused by the complex geography and climatic conditions. High altitudes and low average temperatures at the reproductive stage of rice in Yunnan are ideal natural conditions for screening booting stage cold tolerance in rice germplasm capable of flowering and producing seeds at 17–18°C. KMXBG, cultivated in Kunming for more than 300 years (Cheng 1993), is one of the most low temperature-tolerant landraces at all growth stages, whereas Towada is one of the least tolerant varieties identified in collaborative studies between Japan and China (Horisue *et al.* 1988).

In order to locate QTLs conferring cold tolerance at the booting stage, and ultimately to clone the genes involved, we developed a set of cold-tolerant NILs by backcrossing KMXBG as donor to Towada, and selecting cold-tolerant individuals in each generation of backcrossing (Zhou *et al.* 2010). The objective of the present study was to characterize and identify these cold tolerant NILs at the booting stage. The relationships between agronomic characters and cold tolerance, and the value of SSR markers linked to cold tolerance for potential use in genetic and breeding studies are discussed.

Materials and Methods

Plant materials

Materials used in this study consisted of five BC₆F₅ NILs (1913-4, 1916-1, 1920-5, 1929-4 and 1891-7) developed by backcrossing KMXBG as donor to the cold-sensitive Japanese commercial japonica cv. Towada. In each backcross generation, presumed cold tolerant plants showing normal anthers were selected as male parents under naturally cold conditions in Kunming. In the subsequent selfing generations, cold tolerant plants with the highest spikelet fertilities were selected. The five BC₆F₅ NILs came from the same BC₆F₁ individual.

Characterization of NILs at the booting stage under normal and cold conditions

NILs were individually harvested after growth in two environments, viz. Kunming (low temperature) and Beijing (normal temperature), respectively. Treatment conditions were reported by Zhou *et al.* (2010). Twelve traits of the NILs and parents were investigated at the booting stage; these included plant height (PH), panicle length (PL), internode length below the panicle (NL), panicle exertion (PE), flag leaf length (FLL), flag leaf width (FLW), penultimate leaf length (PLL), penultimate leaf width (PLW), number of full grains per panicle (FG), number of blighted grains per panicle (BG), total grains per panicle (TG) and spikelet fertility (SF) of the main panicle. The significance of differences in these traits between the recurrent parent Towada and each

NIL was determined on the means of 5 plants per line. The mean values of trait differences were compared by t tests using the statistical program SPSS for Windows, version 11.0 (SPSS Inc. 2002).

Evaluation of cold tolerance at different growth stages

The following treatments were applied to evaluate cold tolerance at different growth stages:

(1) Evaluation of cold tolerance at the budding (germination) stage followed protocols adopted at IRRI. To ensure complete dryness and to break any dormancy seeds were placed in a calorstat oven at 45–50°C for 48 h. Ninety well developed seeds of each accession were divided equally, placed in three dishes with wet filter papers, and incubated at 30°C. When shoots were about 5 mm long, the germinating seedlings were subjected to cold treatment at 2°C for 3 days, and then moved to a sunny indoor environment where the temperature was higher than 20°C to ensure normal growth. Seedling survival rates were assessed after 7 days and cold tolerance evaluation indices were determined as: Seedling survival rate = Surviving seedlings/Budding seeds × 100%.

(2) Evaluation of cold tolerance at the seedling stage: Seeds were dried and soaked as above and then germinated at 30°C for 7 days. Fifty uniformly germinating seedlings of each line (1 cm radicle, 0.15 cm plumule) were planted in a ceramic pot and grown to the 3–4 leaf stage at 28°C/24°C, after which they were moved to a 5°C growth cabinet for 7 days. Seedling death rates after six days were used to calculate cold tolerance indices (Li *et al.* 2006).

(3) Evaluation of cold tolerance at the booting stage. Evaluations were carried out in Kunming where the NILs were subjected to 18–21°C for 14 days at the booting stage (Zhou *et al.* 2010). The grading standards for cold tolerance at different growth stages are shown in Table 1.

DNA extraction and molecular marker analysis

DNA was extracted from leaves following the CTAB method (Rogers and Bendich 1988) with minor modifications. A total of 647 SSR markers evenly distributed over all 12 chromosomes (mean interval 2.4 cM, entire genome

Table 1. Grading standards for cold tolerance at different growth stages in rice

Rating	Budding	Seedling	Booting
	Seedling survival rate %	Seedling death rate %	Unfilled grains %
1	≥90	0–20	1–10
2		21–30	11–20
3	80–90	31–40	21–30
4		41–50	31–40
5	50–79	51–60	41–50
6		61–70	51–60
7	1–49	71–80	61–70
8		81–90	71–80
9	0	91–100	81–100

Table 2. Comparisons of cold tolerance-related traits at the booting stage between NILs and Towada under conditions of normal treatment in Beijing and cold treatment in Kunming

Trait	Treatment	Towada	1913-4	1916-1	1920-5	1929-4	1891-7	KMXBG ^a
Plant height (cm)	Normal	104.0	113.4	112.4	104.4	102.2	102.2	/
	Cold	71.7	66.9	70.7	72.5	73.6	65.3	166*
Panicle length (cm)	Normal	18.4	21.7	19.4	21.2	21.3	20.6	/
	Cold	16.8	17.9	16.4	17.9	17.2	14*	26.4*
Inter-node length below the panicle (cm)	Normal	35.3	38.2	35.8	38.5	36.1	37.0	/
	Cold	27.7	28.3	28.8	27.9	29.3	26.7	48.5*
Panicle neck length (cm)	Normal	8.2	8.7	8.2	11.4*	8.0	9.5	/
	Cold	4.4	4.0	5.6	4.3	5.5	4.7	12.9*
Flag leaf length (cm)	Normal	27.9	38.0	32.3	40.4*	33.2	32.8	/
	Cold	23.9	27.4	25.9	27.6	27.1	22.1	25.8
Flag leaf width (cm)	Normal	1.4	1.6	1.6	1.7	1.6	1.7	/
	Cold	1.4	1.3	1.4	1.5	1.3	1.3	1.3
Penultimate leaf length (cm)	Normal	39.2	46.5	40.8	54.3*	47.7	37.3	/
	Cold	28.7	30.8	32.3	33.8	33.4	27.9	45.8*
Penultimate leaf width (cm)	Normal	1.2	1.4	1.4	1.4	1.5	1.3	/
	Cold	1.3	1.1	1.2	1.2	1.2	1.1	1.4
Full grains per panicle	Normal	126.8	121.8	135.0	134.6	135.8	108.0*	/
	Cold	45.3	70.4*	72.2*	72.8*	66.7*	65.3*	188.3*
Blighted grains per panicle	Normal	8.2	7.8	13.7	3.2	14.8	7.6	/
	Cold	71.3	51.0*	29.2*	33.3*	36.8*	40.5*	16.0*
Total grains per panicle	Normal	135.0	129.6	148.7	137.8	150.6	115.6*	/
	Cold	116.6	121.4	101.3	106.0	103.4	105.8	204.3*
Mean spikelet fertility (%)	Normal	93.9	94.0	90.8	97.7	90.2	93.4	/
	Cold	38.8	58.1*	70.4*	66.8*	64.0*	61.3*	91.8*

^a KMXBG did not head under normal treatment conditions in Beijing.

*Significant at P = 0.05.

1,526.8 cM; International Rice Genome Sequencing Project 2005) were assessed for polymorphisms between KMXBG and Towada (McCouch *et al.* 1988, 2002, Temnykh *et al.* 2000). One hundred and eighty three SSR markers showing polymorphisms were used to genotype the NILs. The proportions of genetic background recovery in the NILs were also determined as: $(1 - \text{No. of introgressed markers} / \text{No. of total markers}) \times 100\%$.

Results

Characterization of the NILs

Under normal conditions in Beijing the 12 cold tolerance-related traits were not significantly different among the five NILs and recipient parent, except for grain number per panicle in line 1891-7 which was significantly lower than the other lines (Table 2). Cold treatment in Kunming significantly affected all 12 traits. The NILs and Towada were significantly different only in respect of mean spikelet fertility of the main panicle, full grains per panicle and frequencies of blighted seeds. This confirmed that the NILs had genes for cold tolerance and that spikelet fertility of the main panicle can be used as a cold tolerance index at the booting stage.

Analysis of cold tolerance at NILs at different growth stages

Cold treatment results for the NILs and control varieties at the budding, seedling and booting stages are given in

Table 3. Cold tolerance ratings of NILs at different growth stages

Parents/ NIL	Budding		Seedling		Booting	
	Seedling survival rate %	Rating	Seedling death rate (%)	Rating	Proportion of unfilled grains (%)	Rating
KMXBG	100	1	0.0	1	8.2	1
Towada	26.7	7	50.0	4	61.2	7
1913-4	93.3	1	11.1	1	41.9	5
1916-1	85.0	3	33.3	3	29.6	3
1920-5	43.3	7	50.0	4	33.2	4
1929-4	71.6	5	44.4	4	36.0	4
1891-7	36.7	7	66.7	7	38.7	4

Table 3. KMXBG was the most cold-resistant variety at all stages, with a cold tolerance level of 1. The cold tolerance levels of Towada were low, with indices of 7 for budding and booting, and 4 for the seedling stage. In general the NILs performed significantly better than Towada, but were not as tolerant to cold as KMXBG. The indices for the NILs varied from 3 to 5 at booting, and at the budding and seedling stages, they showed significant differences from each other. For example, at both the budding and seedling stages, line 1913-4 expressed a high level of cold tolerance, but line 1891-7 did not; other lines expressed moderate levels. NILs 1913-4 and 1916-1 had stronger cold tolerance not only at booting, but also at the budding and seedling stages.

Table 4. Polymorphic markers in NILs. K and T refer to the respective parental alleles

Marker	Chromosome	1913-4	1916-1	1920-5	1929-4	1891-7	Reference to previously identified QTL
RM81A	1	T	K	K	K	K	Liu <i>et al.</i> 2003
RM525	2	T	T	K	T	T	Liu <i>et al.</i> 2003, Han <i>et al.</i> 2005
RM1221	3	T	T	K	K	T	
RM518	4	K	T	K	T	T	Xu <i>et al.</i> 2008
RM6770	4	K	T	K	T	K	Xu <i>et al.</i> 2008
RM8213	4	K	T	K	T	T	Xu <i>et al.</i> 2008
RM405	5	K	T	K	T	T	
RM1237	5	T	T	T	T	K	
RM7271	5	K	K	T	T	T	Andaya <i>et al.</i> 2003, Shen <i>et al.</i> 2005
RM4924	6	K	T	T	T	K	
RM3608	7	T	T	K	K	T	Takeuchi <i>et al.</i> 2001, Dai <i>et al.</i> 2004
RM7237	7	T	T	K	K	T	Takeuchi <i>et al.</i> 2001, Dai <i>et al.</i> 2004
RM6432	7	T	T	K	K	T	Takeuchi <i>et al.</i> 2001, Dai <i>et al.</i> 2004
RM331	8	T	T	H*	H	H	
RM409	9	K	T	T	K	T	
RM215	9	K	T	T	K	T	Liu <i>et al.</i> 2003
RM552	11	T	T	K	K	T	
RM536	11	T	K	K	T	T	Liu <i>et al.</i> 2003, Oh <i>et al.</i> 2004
RM5338	12	K	K	K	T	K	Dai <i>et al.</i> 2004, Han <i>et al.</i> 2005

* H, heterozygous

Genotypic analysis of NILs

Among 647 SSR markers equally distributed on all chromosomes, 183 were polymorphic between KMXBG and Towada. The results of genotyping of the five NILs with the 183 markers are summarized in Table 4. Nineteen of the 183 markers had alleles from KMXBG, and NILs 1913-4, 1916-1, 1920-5, 1929-4 and 1891-7 had 9, 4, 13, 9 and 7 introgressed markers, respectively. The proportions of genetic background recovery were 98.0–99.4%. Among 19 loci, KMXBG alleles of RM81A and RM5338 were present in four NILs; those of RM6770 and RM331 were in three NILs; those of RM525 and RM1237 were in one NIL; and 13 were each present in two NILs (Table 4).

Discussion

Value of NILs with cold tolerance at the booting stage

Cold tolerance at booting in rice is a complex trait. Because it is difficult to evaluate accurately, there are few reports on QTL mapping of genes controlling this trait. Saito *et al.* (2001, 2004) reported that cold tolerance at booting was not controlled by major genes. Spikelet fertility is generally used as an index of cold tolerance, but this trait is also affected by environment and other cold tolerance-related genes, such as those controlling plant height, panicle neck length and heading date. To reduce the affects of genetic background and to avoid interactions between multiple cold tolerance-related genes, the development of cold tolerant NILs was an appropriate strategy for mapping and eventual cloning of the genes.

In order to avoid hybrid sterility, a common occurrence in inter-subspecies rice crosses, but to exploit a distant geographical relationship, we earlier developed cold-tolerant

NILs using japonica cultivars KMXBG and Towada from Yunnan and Japan (Zhou *et al.* 2010). KMXBG is one of the most cold-tolerant landraces at all growth stages, whereas Towada is one of the least tolerant varieties identified during collaborative studies between Japan and China (Horisue *et al.* 1988). In the present study there were no significant differences in most of the investigated traits between the NILs and Towada when they were grown under normal temperature conditions in Beijing (Table 2). Under the cold conditions in Kunming the only significant differences were for three grain-related traits, viz. full grains per panicle, blighted grains per panicle and mean spikelet fertility (Table 2). These results indicated that the NILs was similar to Towada except for traits associated with spikelet fertility of the main panicle. The effects of other cold-related traits on spikelet fertility were eliminated in the genetic backgrounds of the five NILs. Thus spikelet fertility was an appropriate index to evaluate cold tolerance at the booting stage in segregating populations from NIL × Towada crosses. According to the genotypic analysis, each NIL contained multiple introgressed segments, which probably confirms the complexity of booting stage cold tolerance. These NILs can be used as parents in further crosses to develop sub-NILs.

KMXBG is extremely cold-tolerant at the budding, seedling and booting stages (Table 3). When the five booting stage cold-tolerant NILs were compared with Towada, 1913-4 and 1916-1 displayed enhanced cold-tolerance at the budding and seedling stages; both had the same two alleles, RM7271 on chromosome 5 and RM5338 on chromosome 12, from KMXBG. Notably, the RM7271 allele in these two NILs was not present in the other three, which showed weaker cold tolerances at the budding and seedling stages. In previous studies, the RM7271 locus was overlapped by

cold-tolerance QTLs detected at the booting (Andaya and Mackill 2003) and seedling (Zhan *et al.* 2004) stages. This suggests that RM7271 is near an important cold-tolerance locus expressed at multiple growth stages. Fine mapping and eventual cloning should determine whether there is a pleiotropic effect or if closely linked genes affect cold tolerance at different growth stages.

Correlation analysis of cold tolerance at different growth stages in rice

Cold damage in rice can occur at all growth stages. The amount of damage depends on temperature, cold duration, rice ecotype and variety. Li *et al.* (1981) evaluated cold tolerance in 50 accessions from IRRI at the budding, seedling, booting and flowering stages and showed that cold tolerance was significantly correlated between the budding and seedling, budding and flowering, and seedling and flowering stages, but there were no significant correlations between the other stages. Bertin *et al.* (1996) considered that cold tolerance at the 2 and 8 leaf stages were positively correlated. There were also conflicting results. For example, Chung (1979) reported that correlations of cold tolerance between the seedling and reproductive stages were not significant. In the present study, the correlation of cold tolerance between the budding and seedling stages ($r=0.866$, $P=0.011$) was significant, whereas those between budding and booting ($r=0.529$, $P=0.222$), and seedling and booting ($r=0.309$, $P=0.500$) were not significant. Although KMXBG was a consistently cold tolerant variety, the cold tolerances of the NILs varied between growth stages. For example, 1913-4 had strong cold tolerance at the budding and seedling stages, but only medium tolerance at booting. Our results suggested both coincident and specific mechanisms of cold tolerance at different stages.

Analysis of cold tolerance loci in the NILs

If a locus is not linked with cold tolerance, the probability of the presence of a random homozygous KMXBG allele would be less than 1% after six backcrosses and four selfings while selecting for cold tolerance in each generation ($\left(\frac{1}{2}\right)^6 \times \left(\frac{1}{2}\right) = 0.0078$). Due to genetic hitch-hiking effects (Harr *et al.* 2002, Smith *et al.* 1974) the loci in the five NILs having KMXBG alleles were likely linked with cold tolerance, especially loci with the same KMXBG alleles present in two or more NILs. These loci were distributed on all chromosomes except chromosome 10, but no locus with a KMXBG allele was present in all five NILs. Previous studies (Futsuhara and Toriyama 1966, Saito *et al.* 2004, Zhou *et al.* 2010) showed that cold tolerance in rice at booting is a complex quantitative trait. The present results reconfirm it, and indicate that further detailed analysis of cold tolerance using the NILs would be worthwhile.

Some loci, including those on chromosomes 1, 2, 4, 5, 7, 9, 11 and 12 were in QTL regions detected in previous studies (Table 4). Thus the results appear to be credible. Other

loci which were not detected in previous reports may be new QTL sites. It was interesting that the RM331 locus remained heterozygous in three NILs, despite four selfing generations. It is possible that this locus may be linked to a heterotic cold tolerance gene. NIL 1929-5 (named ZL1929 in the previous report) was chosen to develop a segregating population, and QTL *qCTB7* was eventually fine mapped to a 92-kb interval (Zhou *et al.* 2010). The NILs reported here should be suitable materials for validating and eventual cloning of the cold tolerance genes. Towada is a commercial variety with high grain quality. The cold tolerant Towada NILs should also be useful materials for breeding.

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